THE SECRET COVENANT
An illusion it will be, so large, so vast it will escape their perception.
Those who will see it will be thought of as insane.
We will create separate fronts to prevent them from seeing the connection between us.
We will behave as if we are not connected to keep the illusion alive.
Our goal will be accomplished one drop at a time so as to never bring suspicion upon ourselves.
This will also prevent them from seeing the changes as they occur.
We will always stand above the relative field of their experience for we know the secrets of the absolute.
We will work together always and will remain bound by blood and secrecy.
Death will come to he who speaks.
We will keep their lifespan short and their minds weak while pretending to do the opposite.
We will use our knowledge of science and technology in subtle ways so they will never see what is happening.
We will use soft metals, aging accelerators and sedatives in food and water, also in the air.
They will be blanketed by poisons everywhere they turn.
The soft metals will cause them to lose their minds.
We will promise to find a cure from our many fronts, yet we will feed them more poison.
The poisons will be absorbed trough their skin and mouths, they will destroy their minds and reproductive systems.
From all this, their children will be born dead, and we will conceal this information.
The poisons will be hidden in everything that surrounds them, in what they drink, eat, breathe and wear.
We must be ingenious in dispensing the poisons for they can see far.
We will teach them that the poisons are good, with fun images and musical tones.
Those they look up to will help.
We will enlist them to push our poisons.
They will see our products being used in film and will grow accustomed to them and will never know their true effect.
When they give birth we will inject poisons into the blood of their children and convince them its for their help.
We will start early on, when their minds are young, we will target their children with what children love most, sweet things.
When their teeth decay we will fill them with metals that will kill their mind and steal their future.
When their ability to learn has been affected, we will create medicine that will make them sicker and cause other diseases for which we will create yet more medicine.
We will render them docile and weak before us by our power.
They will grow depressed, slow and obese, and when they come to us for help, we will give them more poison.
We will focus their attention toward money and material goods so they many never connect with their inner self.
We will distract them with fornication, external pleasures and games so they may never be one with the oneness of it all.
Their minds will belong to us and they will do as we say.
If they refuse we shall find ways to implement mind-altering technology into their lives.
We will use fear as our weapon.
We will establish their governments and establish opposites within.
We will own both sides.
We will always hide our objective but carry out our plan.
They will perform the labor for us and we shall prosper from their toil.
Our families will never mix with theirs.
Our blood must be pure always, for it is the way.
We will make them kill each other when it suits us.
We will keep them separated from the oneness by dogma and religion.
We will control all aspects of their lives and tell them what to think and how.
We will guide them kindly and gently letting them think they are guiding themselves.
We will foment animosity between them through our factions.
When a light shall shine among them, we shall extinguish it by ridicule, or death, whichever suits us best.
We will make them rip each other's hearts apart and kill their own children.
We will accomplish this by using hate as our ally, anger as our friend.
The hate will blind them totally, and never shall they see that from their conflicts we emerge as their rulers.
They will be busy killing each other.
They will bathe in their own blood and kill their neighbors for as long as we see fit.
We will benefit greatly from this, for they will not see us, for they cannot see us.
We will continue to prosper from their wars and their deaths.
We shall repeat this over and over until our ultimate goal is accomplished.
We will continue to make them live in fear and anger though images and sounds.
We will use all the tools we have to accomplish this.
The tools will be provided by their labor.
We will make them hate themselves and their neighbors.
We will always hide the divine truth from them, that we are all one.
This they must never know!
They must never know that color is an illusion, they must always think they are not equal.
Drop by drop, drop by drop we will advance our goal.
We will take over their land, resources and wealth to exercise total control over them.
We will deceive them into accepting laws that will steal the little freedom they will have.
We will establish a money system that will imprison them forever, keeping them and their children in debt.
When they shall ban together, we shall accuse them of crimes and present a different story to the world for we shall own all the media.
We will use our media to control the flow of information and their sentiment in our favor.
When they shall rise up against us we will crush them like insects, for they are less than that.
They will be helpless to do anything for they will have no weapons.
We will recruit some of their own to carry out our plans, we will promise them eternal life, but eternal life they will never have for they are not of us.
The recruits will be called "initiates" and will be indoctrinated to believe false rites of passage to higher realms.
Members of these groups will think they are one with us never knowing the truth. They must never learn this truth for they will turn against us.
For their work they will be rewarded with earthly things and great titles, but never will they become immortal and join us, never will they receive the light and travel the stars.
They will never reach the higher realms, for the killing of their own kind will prevent passage to the realm of enlightenment.
This they will never know.
The truth will be hidden in their face, so close they will not be able to focus on it until its too late.

Oh yes, so grand the illusion of freedom will be, that they will never know they are our slaves.
When all is in place, the reality we will have created for them will own them.
This reality will be their prison.
They will live in self-delusion.
When our goal is accomplished a new era of domination will begin.
Their minds will be bound by their beliefs, the beliefs we have established from time immemorial.
But if they ever find out they are our equal, we shall perish then.
THIS THEY MUST NEVER KNOW.
If they ever find out that together they can vanquish us, they will take action.
They must never, ever find out what we have done, for if they do, we shall have no place to run, for it will be easy to see who we are once the veil has fallen.
Our actions will have revealed who we are and they will hunt us down and no person shall give us shelter.
This is the secret covenant by which we shall live the rest of our present and future lives, for this reality will transcend many generations and life spans.
This covenant is sealed by blood, our blood. We, the ones who from heaven to earth came.
This covenant must NEVER, EVER be known to exist.
It must NEVER, EVER be written or spoken of for if it is, the consciousness it will spawn will release the fury of the PRIME CREATOR upon us and we shall be cast to the depths from whence we came and remain there until the end time of infinity itself.

Cannabis Research A to Z

https://www.ncbi.nlm.nih.gov/pmc/?term=endocannabinoid+system+and+cocaine+dependence&cmd=DetailsSearch

endocannabinoid system and cocaine dependence
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1906: The Rothschilds' claim that due to growing instability in the region and increasing competition from Rockefeller (the Rockefeller family are Rothschild descendants through a female bloodline) owned Standard Oil, they decide to sell their Caspian and Black Sea Petroleum Company to Royal Dutch and Shell.
This is another example of the Rothschilds' trying to hide their true wealth which they are actually consolidating.
The Rockefeller family (/ˈrɒkəfɛlər/) is an American industrial, political, and banking family that made one of the world's largest fortunes in the oil business during the late 19th and early 20th centuries, with John D. Rockefeller and his brother William Rockefeller primarily through Standard Oil.[1]
The family is also known for its long association with and control of Chase Manhattan Bank.[2]
They are considered to be one of the most powerful families, if not the most powerful family,[3] in the history of the United States.
One of the founding members of the Rockefeller family was businessman William Rockefeller Sr. born in Granger, New York, to a Protestant family.
He had six children with his first wife Eliza Davison, the most prominent of which were oil tycoons John Davison Rockefeller and William Rockefeller, co-founders of Standard Oil.
Oil baron John D. Rockefeller was a devout Northern Baptist, and he supported many church-based institutions.
The combined wealth of the family – their total assets and investments plus the individual wealth of its members – has never been known with any precision.
The records of the family archives relating to both the family and individual members' net worth are closed to researchers.[19]
From the outset, and even today, the family's wealth has been under the complete control of the male members of the dynasty, through the family office.
Despite strong-willed wives who had influence over their husbands' decisions—such as the pivotal female figure Abby Aldrich Rockefeller, wife of Junior—in all cases they received allowances only and were never given even partial responsibility for the family fortune.
Much of the wealth administered by Chase Bank has been locked up in the notable family trust of 1934 (which holds the bulk of the fortune and matures on the death of the fourth generation), and the trust of 1952, both administered by Chase Bank, the corporate successor to Chase Manhattan Bank. These trusts have consisted of shares in the successor companies to Standard Oil and other diversified investments, as well as the family's considerable real estate holdings.
They are administered by a trust committee that oversees the fortune.
Management of this fortune today also rests with professional money managers who oversee the principal holding company, Rockefeller Financial Services, which controls all the family's investments, now that Rockefeller Center is no longer owned by the family.
The present chairman is David Rockefeller Jr.
In 1992, it had five main arms:
Rockefeller & Co. (Money management: Universities have invested some of their endowments in this company);
Venrock Associates (Venture Capital: an early investment in Apple Computer was one of many it made in Silicon Valley entrepreneurial start-ups);
Rockefeller Trust Company (Manages hundreds of family trusts);
Rockefeller Insurance Company (Manages liability insurance for family members);
Acadia Risk Management (Insurance Broker: Contracts out policies for the family's vast art collections, real estate and private planes.)[21]
In September 2014, the Rockefeller Brothers Fund announced it will divest its investments in fossil fuel companies.[22]
In March 2015, the chair of the Rockefeller Brothers Fund stated two conclusions, one that; "it's immoral to continue down the fossil fuel path", and secondly that it is "financially imprudent to stay invested in companies whose profits depend on defying ... the international effort to restrain climate change.
The F.D.A In this video /read me by Anonymous Hacking Group] we will expose the ties between the Rockefeller family chemistry & the pharmaceutical industry.
The Food & Drug administration began life as the Division of the Bureau of Chemistry, long before changing its name to the F.D.A. Food & Drug Administration.
It is believed that its name was changed to conceal its chemical industry agenda to blur the dividing line between what is good & what isn't & fulfill Global eugenics sorcery chemical depopulation.
It is always provided a type of legal immunity for companies that put chemicals into foods, and to pharmaceutical companies via so called its approvals"; which leave citizens without legal recourse against the chemical industry.
these are the original chemists from the Bureau of Chemistry, in 1909 -- that later become the F.D.A.. Bureau of the Food & Drug Administration.
It's true job was, and is, to "approve" and sanction products from the chemical industry, and thereby legally shield the chemical industry from us by declaring what is "safe" & what isn't.
They routinely privately gather data to share with their chemical industry partners to mitigate liability.
For example, contamination information that they hide from the public.
These are "P.R.-public relations excuse issues" for them.
Good luck winning a case in a U.S. United States Court against a billion dollar chemical company whose products are "F.D.A. approved" and given a G.R.A.S. (Generally' Recognized As Safe) SAFE HARBOR STATUS.
These shield the chemical companies, and give them plausible deniability.
The modern F.D.A. came into being in 1913 -- the same year that the Rockefeller Foundation was created.
The F.D.A. works hand-in-hand with the Rockefeller Foundation & the American Medical Association.
The Rockefeller Foundation was founded in 1913, with it's main emphasis upon medicine & medical EDU education.
The initial Rockefeller medical school donations totaled over $550,000,000. in 1913 alone.
The Rockefeller Foundation gave money to 18 medical schools across 14 countries.
It's partners at the F.D.A. began an aggressive campaign of suppressing medicines that competed with the chemical industry.
Dr Richard J. Crout, Director of the F.D.A. Bureau of Drugs said in Spotlight on January 18, 1982, I never have & never will approve a new drug to an individual, but only to a large pharmaceutical company with unlimited finances."
Suppressed were the natural medicines that their industry could not legally control & monopolize with "drug" (chemical) patents.
Natural substances can never be patented.
This is why every natural and non-toxic therapy is automatically called "quackery" by the F.D.A., the chemical industry & the medical establishment.
Only unnatural (ie, non-organic toxic), from CRUDE OIL/TAR petrochemicals may be patented, & thus, non-toxic medicines are a threat to the corupped business of poison under the name of medicine.
A report from the McGill University Press Said in 1919, a five million dollar Rockefeller Foundation gift to certain Canadian medical schools helped bring Canadian medical education to the 20th century.
Medicine was not the lucrative market that it is today, so medical schools were begging for the Rockefeller grants that were being handed out to COMPLIANT SCHOOLS.
COMPLIANCE to the Rockefeller stipulations required abandoning traditional & natural medicines in lieu of a new genocidic generation of BIO petrochemicals for drugs, & trashing ENTIRE LIBRARIES of past procedures.
What happened was a preamble to book burnings, elsewhere, and in some cases, the F.D.A. did indeed have book burnings.
Those were dangerous times for traditional naturopathic (non-chemical industry) doctors.
All dissenters who embraced the old time-tested holistic & naturopathic methodologies of healing would henceforth be demonized as "quacks" in formal medical training.
That was despite some of those "quacks" having Nobel Prizes in medicine, & vastly superior results.

It was a carrot & stick strategy. compliant schools were given the carrot (Rockefeller money).
Competing doctors were given the stick (F.D.A. police actions).
Some Doctors ended up dead-were killed or sentenced to life in prison for having cured to many patients with "unapproved" medicine.
It is still happening.
Don't you feel like being rapped by Rockefeller's F.D.A. by reading this?
At stores selling organic (non toxic-chemical) foods.
certain cancer-killing vitamins or even raw milk. it is not just the doctors with cures anymore.
Curing is illegal, by the way, since no cures are ever approved".
Cures mean freedom from chemical dependence: freedom from City Hall, Rockefeller, DuPont, D.E.A. 6630507, F.D.A. 201(ff)(3)(B)(ii), A.C.S, the N.I.C., the A.M.A. & the corrupt major oncology centers.
An unholy alliance formed between the American Medical Association, the F.D.A., & the Rockefeller Foundation to insure compliance from the Medical schools.
The Rockefeller Foundation Frequently insisted that medical schools place Rockefeller employees on their board of directors.
The general Education Board of the Rockefeller Foundation, also approached in 1914, pledged $5000,000 on the condition that the University raised $1.5,000,000 by January 1, 1916 & paid clinical faculty [allopathic doctors only] on a full-time basis.
With the Bribed money and the hope of GEB funding, the Yale Corporation & Hospital signed the affiliation contract with the Hospital before the deadline of July 1, 1914." --
Yale University Medical School Web Site.
The new legally enforceable medical monopoly paralleled the past Rockefeller monopoly from the petro, bio CRUDE OIL/TAR chemical industry of times past. Instead of owning all petroleum, the Rockefeller SATANIC Empire now controlled virtually all POISONOUS medicine whereby the Rockefeller can now sell many petroleum POISONOUS chemicals, (POISONS in fact) that cannot be utilize as fuel, plastics or synthetics.
He owned the lion's share of the chemical industry, which would later be called the "pharmaceutical" industry.
Throughout his life, John D. Rockefeller, Sr. refused to take his own medicine, for it is POISON.
He used traditional holistic medicines for his own health his entire life. Alongside many of his friends at the F.D.A. & the A.M.A..
After the hijack of the medical schools, the true carnage of polio, heart disease & cancer exploded.
"I had a brain cancer specialist sit in my living room & tell me that he would never take radiation if he had a brain tumor, & I asked him, but, do you send people for radiation?' & he said; of course, I'd be drummed out of the hospital if I didn't,'" Said Dr Ralph Moss, Ph.D. winner of 1937 Nobel prize of medicine
Gyorgyi, Ph.D., discoverer of vitamin C, winner of 1937 Nobel Prize for medicine said "The American Cancer Society tried to ruin my research foundation. Guess when this organization [American Cancer Society) was founded & by whom.
It was founded by John D. Rockefeller Jr. in that MAGIC year 1913, while "donating" at Harvard University.
Do you believe that they will someday announce a cure, & cut themselves off from the truckloads of money?
A better question would be why vitamin C. was a threat to their mission.
What is their maine aim again, oh yah; DEPOPULATION while Gaining the whole World for themselves.
According to the U.S. Census, cancer is the 2nd largest killer in the United States, but most of those people die from the POISONS, ah I meen medicine's; not the cancers.
Pharmaceutical drugs are the fourth largest killer.
The U.S. has the most dangerous drugs, & yet the most expensive medical system in the World, it causes 60% of all U.S. bankruptcies.
Dr. Robert Atkins, M.D., (creator of "the Atkins Diet") said "There is not one, but many Cures for cancer available, but they are all being systematically suppressed by the A.C.S, the N.I.C. & the major oncology centers.
They have too much of an interest in the status quo."
Cancer cure Vitamin B17. (laetrile. In 2007 bitter almonds (not regular almonds) were becoming popular with alternative medicine due to their vitamin B17. content.
Health food stores stopped selling B17. in the form of apricot seeds in 2002, due to armed F.D.A raids.
There were reports everywhere of cancers being killed merely from patients eating heaping servings of bitter almonds daily.
Soon there would be almond extract capsules, so that these same patients would need only to take one cheap, non-toxic pill per day.
It did not bode well for the pharmaceutical industry.
Hundreds of billions of dollars were about to be lost.
The F.D.A. openly banned apricot seeds, & the vitamin B17. extract, because it was successfully curing cancers as an "unapproved drug".
The British Government soon followed suit, & did the vary same thing during 2002.
people in both countries were arrested & charged for merely selling organic apricot seeds, a carrier of unapproved vitamin B17. (laetrile).

Similar to a year ago, when the Food & Drug Administration (FDA) issued warning letters to six companies, the FDA issued several warning letters to marketers of products with cannabidiol (CBD):
ABC Productions
BREAKING: FDA Issues Warning Letters to 8 Marketers of CBD Products
The letters are all fairly similar and criticize health claims made by the company as supplements.
Unless the product was marketed before GW Pharma (NASDAQ: GWPH) published clinical trials, the companies can’t make health claims:
FDA has concluded that CBD products are excluded from the dietary supplement definition under section 201(ff)(3)(B)(ii) of the Act [21 U.S.C. § 321(ff)(3)(B)(ii)].
Under that provision, if a substance (such as CBD) has been authorized for investigation as a new drug for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public, then products containing that substance are outside the definition of a dietary supplement.
There is an exception if the substance was “marketed as” a dietary supplement or a conventional food before the new drug investigations were authorized; however, based on available evidence, FDA has concluded that this is not the case for CBD.
Note that this is a very broad attack on the CBD-from-hemp industry and includes products from CannaVest (OTC: CANV) as well as other companies like Endoca and Bluebird Botanicals.

HEALTH FREEDOM ALERT: The FDA assaults CBD extracts, attempts to regulate out of existence but industry pushes back (CORRECTION)
by Mike Adams, the Health Ranger
(NaturalNews) MAJOR CORRECTIONS: I'm issuing a huge correction / clarification to this story after speaking with people in the hemp industry. They've pointed out that my article, although posted with the best intentions to help keep CBDs legal and readily available, was freaking out CBD retailers who thought some sort of new law had been passed outlawing CBDs.
In no way did I intend to disrupt CBD retailers with that article!
My only goal was to sound the alarm of how to FDA is attempting to destroy this entire industry, and help create a backlash that would keep natural medicine legal in America.
The FDA is trying to destroy the CBD industry with regulatory assaults, but the industry is pushing back (and the weight of the evidence and the science is on the side of the hemp industry).
Nevertheless, the FDA is a formidable opponent and they can wage all sorts of regulatory assaults against natural substances they wish to regulate out

of existence.
So I've decided to post a serious correction / clarification to this story to make sure nobody misreads what I'm asserting here, and I apologize for any misconstruing of my intention in posting this, which was to ALERT people to the regulatory assaults of the FDA that are trying to destroy the hemp extract industry.
As a food scientist and natural medicine activist, I am 100% pro hemp, pro CBDs and pro plant medicine.
Essentially, my use of the term "outlawed" in the title of my original article was misconstrued. By "outlawed," I meant the FDA essentially writes its own "regulatory laws" and has posted a clear statement on its own website which states that CBDs cannot be sold as a dietary supplement.
This is a regulatory attack on hemp extracts, but it is not technically a law that was passed by Congress.
I regret my choice of the word "outlawed", and I will be more careful in my choice of words when covering this issue in the future.
In the article below, I have replaced any reference to "outlawing" with the more accurate terminology of "regulating out of compliance" or similar language.
But make no mistake: Cannabidiol is on the target radar of the FDA, and they are waging a campaign of regulatory intimidation to try to destroy the marketplace.
If they get their way, they will EFFECTIVELY outlaw CBDs via regulatory assaults.
Their goal is the same: destroy the CBD marketplace.
My hope is that they fail in their efforts, of course.
Original story follows, with corrections as noted above
Hemp oil extracts containing CBDs (cannibidiols) are such a threat to the pharmaceutical industry that the FDA is now invoking totally insane justifications for outlawing them.
UPDATE: This article has caused a tremendous amount of discussion and a lot of disinformation is flying around, so I'm adding these notes for clarification.
One thing I've learned is that the people who sell CBD oil don't even know there is more than one CBD molecule.
Some have attacked me for using "cannabidiols" in a plural form, because they are ignorant of the full chemistry of CBD synthesis and molecular variants.
Even wikipedia lists seven CBD molecular variations, each with its own name.
They are named delta-5-cannabidiol, delta-4-cannabidiol, delta-3-cannabidiol, delta-3,7-cannabidiol, delta-2-cannabidiol, delta-1-cannabidiol and delta-6-cannabidiol.
They share the same elements in their molecular composition, but they have variations in their double bonds, meaning they behave slightly differently when interacting with other molecules.
Secondly, the FDA did attempt to "regulate into non-compliance" CBDs as a dietary supplement.
It's printed right on the FDA's own website: "12.
Can products that contain cannabidiol be sold as dietary supplements?
A. No.
Based on available evidence, FDA has concluded that cannabidiol products are excluded from the dietary supplement definition..."
The FDA has also started sending aggressive warning letters to CBD product marketers.
However, the industry is rightly pushing back against this unjustified regulatory classification by the FDA.
In fact, the industry needs to push back even harder, or else the FDA will steamroll everyone...
Some of the people commenting on this article have said you can just sell CBDs as a "food" and not a dietary supplement.
UPDATE: Efforts are under way to GRAS list cannabidiol with the FDA, but CBD is not currently GRAS listed, meaning it cannot be sold as a food without attracting regulatory scrutiny from the FDA.
I spoke with a highly competent regulatory attorney who confirmed all this.
There is no question that some smaller companies are right now selling hemp oil with CBD content, but this does not mean such sales are immune from FDA regulatory prosecution (and intimidation attempts).
The FDA tends to go after larger suppliers first, ignoring smaller suppliers due to its own limited resources.
For this reason, smaller suppliers of CBD supplements or hemp oil products containing added CBDs may be able to continue selling such products for a very long time without facing regulatory action.
Nevertheless, that regulatory risk is ever present and may be invoked at any time by the FDA to target that company and confiscate its products.
Selective prosecution is one of the key regulatory weapons of all federal agencies.
This is how they protect their pharma friends and punish the competition.
This is why the hemp industry needs to push back HARD against the FDA!
CBDs are non-psychoactive compounds found naturally in hemp plants.
They work so well as powerful natural medicine that people everywhere are realizing CBDs work better than pharmaceuticals for treating epilepsy, seizures, neurological disorders and other serious health conditions (including HIV infections).
So the FDA has just launched a massive regulatory assault against CBDs by invoking the most insane logic you've ever heard.
Here's how it goes:
1) CBDs work so well that drug companies are now investigating them to be approved by the FDA as medicines.
When the FDA considers a substance to be 'authorized for investigation as a new drug' if it is the subject of an Investigational New Drug application (IND) that has gone into effect," for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public, then products containing that substance are outside the definition of a dietary supplemen.
The FDA is now attempting to regulate every natural plant molecule: (vitamins, minerals, enzymes, & exogenous phyto-hormone related compounds) that prevents, treats, or curse disease out of existence as dietary supplements.
The FDA's own website: "FDA has concluded that cannabidiol products are excluded from the dietary supplement definition under section 201(ff)(3)(B)(ii) of the FD&C Act. 4)
Now the FDA has begun sending warning letters to CBD makers, claiming they are in violation of FDA regulations because they are selling "adulterated products."
Adulterated with what, exactly? CBDs, of course!
"The debate over hemp CBD’s legal status continues after the FDA sent eight warning letters to manufacturers of CBD dietary supplement and food products."
Reports Nutritional Outlook.
"The warning letters cite impermissible health claims used to market the products, as well as CBD’s invalid status as a dietary ingredient due to its presence in two drug applications currently under consideration."
"Drugs," according to the FDA, are substances that are proven to treat, prevent or cure a disease: (nutrition)
But when a dietary supplement company makes the same claim, they are hit with aggressive warning letters from the FDA, threatening to shut them down, confiscate their products and seek criminal prosecution of the company executives.
This is how the FDA can instantly criminalize any vitamin or plant extract. Do you see how this twisted, corrupt regulatory tactic can allow the FDA to instantly criminalize any dietary supplement, vitamin, herb or plant extract?
Vitamin D, for example, could be outlawed by the FDA announcing that it has granted "Investigational New Drug application (IND)" status to any drug company studying vitamins under the pharmaceutical drug defamation.
Using this same corrupt, mafia-style tactic, the FDA could essentially "outlaw" resveratrol or even vitamin C, denying Americans the right to access

safe, affordable, natural substances that are routinely found in nature.
It's time to end the FDA's monopolization over natural plant molecules.
Any time a healing substances is found in nature, the FDA can simply assert that such molecules are now in the process of being studied as drugs and are therefore illegal to sell as dietary supplements even though they are recurring in Nature, not drug companies.
This FDA tactic is nothing more than the FDA proclaiming intellectual monopolies over all molecules of interest found in nature.
It is Monsanto-like in its arrogance and wickedness, and it deprives the People of their right to access medicinal plants and healing substances that have existed in nature for thousands of years long before the FDA ever came into existence in(1906).
If we do not stop this FDA, we are all doomed to living under a totalitarian regulatory regime that will systematically criminalize every healing molecule found in nature, from the curcuminoids in turmeric root to green tea catechins and even phycocyanins found in spirulinam.
That this federal agency has now resorted to such tactics of medical totalitarianism and outright tyranny is proof that we need to strip the FDA of regulatory powers over natural dietary supplements and limit its domain to pharmaceutical drugs only And reinstate the use of industrial HEMP and raw cannabis as a food source.
A 60 gram supply of cannabis oil is recommended for those suffering from serious disease such as cancer.
Each tube is 10 grams.
pictured here is enough to treat one cancer patient & only $600 to 1,200 total.
The health care industry would charge you that much for the first night in the hospital.
The United States Government manipulated by the people to hide the federal name, has known since 1974 that cannabis kills cancer.
In 1972 Richard Nixon wanted a larger budget for his war on drugs.
He thought if he could prove that cannabis damages the immune system causing lung cancer like cigarettes do, he would get the support he needed.
He gave the Medical College of Virginia, who had been funded by the national institute of health 2 years to do the study on the effects of THC on the body.
In 1974 the study was completed, it turns out they found instead when THC is ingested in highly concentrated forms, such as eating cannabis oil will attack any mutated cells in your body, why'll strengthening & rejuvenating the healthy cells.
The researchers found instead that THC slowed the growth of three kinds of cancer in mice.
they found the perfect cure for cancer.
It worked fast. It worked well, it worked on many forms of cancer in all stages & it had zero harmful side effects unlike chemotherapy which deteriorates the entire body which is known to kill 1 in 5 patients.
Not only can cannabis cure cancer, it also has been proven that it can dissolve all forms of tumors & can even combat super bugs like MRSA.
When Richard Nixon saw the results of the study, he got furious.
He through the entire report in the trash & deemed the study classified.
In 1976 president Gerald ford had the DEA quickly shut down the Virginia study and all further public cannabis tumor research and granted exclusive research rights to major pharmaceutical companies who set out unsuccessfully to develop synthetic forms of THC that would deliver all the medical benefits without the so called high.
since they were unable to develop a synthetic form of THC that was as good as natural real THC, Ronald Reagan commissioned Dr. Heath/Tulane University to kill Rhesus monkies with carbon monoxide poisoning
Damage and Dead Monkeys!
In 1974, California Governor Ronald Reagan was asked about decriminalizing marijuana.
After producing the Heath/Tulane University study, the so-called "Great Communicator" proclaimed, "The most reliable scientific sources say permanent brain damage is one of the inevitable results of the use of marijuana." (L.A. Times)
The report from Dr. Heath had concluded that Rhesus monkeys, smoking the equivalent of only 30 joints a day, began to atrophy and die after 90 days.
And ever since, dead brain cells found in monkeys who were forced to smoke marijuana has been given maximum scare play in federal booklets and government sponsored propaganda literature against pot.
Senator Eastland of Mississippi used it throughout the mid-1970s to horrify and stop national legislators from supporting NORML's decriminalization bills in Congress, mostly sponsored by the late Senator Jacob Javitts of New York.
Reports of the study have also been distributed by the hierarchy of drug rehabilitation professionals as part of their rationalization for wanting to get kids off pot, based on supposed scientific studies.
It is used to terrorize parent groups, church organizations, etc., who redistribute it still further.
Heath killed the half-dead monkeys, opened their brains, counted the dead brain cells, and then took control monkeys, who hadn't smoked marijuana, killed them too, and counted their brain cells.
The pot smoking monkeys had enormous amounts of dead brain cells as compared to the "straight" monkeys.
The public outlook"
Ronald Reagan's pronouncement by trusting the federal research to be real and correct was based on the fact that marijuana smoking was the only difference in the two sets of monkeys.
The reality is Reagan had other motives. Ronald Reagan was part of the brotherhood, “a 33d% Lucifarian Freemason.”
Hemp was a threat to their illegal Federal Reserve, drug trades, cruid oil, petrochemical, pharmiceutical, cancer, synthetic, current agriculture, lumber, cotton, tobacco and automobile endustries and the defamation of corperate America as a whole.
The only way to keep their corupped running intrest was to clasify Hemp as the moat deadly substance under schedule 1.
The government ballyhooed false science to press and PTA, who trusted the government completely.
In 1980, Playboy and NORML finally received for the first time after six years of requests and suing the government an accurate accounting of the research procedures used in the infamous report:
When NORML/Playboy hired researchers to examine the reported results against the actual methodology, they laughed.
The Facts:
Suffocation of Research Animals
As reported in Playboy, the Heath "Voodoo" Research methodology involved strapping Rhesus monkeys into a chair and pumping them with equivalent of 63 Colombian strength joints in "five minutes, through gas masks," losing no smoke.
Playboy discovered that Heath had administered 63 joints in five minutes over just three months instead of administering 30 joints per day over a one-year period as he had first reported.
Heath did this, it turned out, in order to avoid having to pay an assistant's wages every day for a full year.
The monkeys were suffocating! Three to five minutes of oxygen deprivation causes brain damage"dead brain cells."
(Red Cross Lifesaving and Water Safety Manual) With the concentration of smoke used, the monkeys were a bit like a person running the engine of a car in a locked garage for 5, 10, 15 minutes at a time every day!
The Heath Monkey study was actually a study in animal asphyxiation and carbon monoxide poisoning.
Among other things, Heath had completely (intentionally? incompetently?) omitted discussion of the carbon monoxide the monkeys inhaled.
Carbon monoxide, a deadly gas that kills brain cells, is given off by any burning object.
At that smoke concentration, the monkeys were, in effect, like a person locked in a garage with the car engine left running for five, 10, 15 minutes at a time every day!
All subsequent researchers agree the findings in Heath's experiment regarding marijuana were of no value, because carbon monoxide poisoning and other factors were totally left out and had not been considered in the report.

This study and others, like Dr. Gabriel Nahas' 1970s studies, tried to somehow connect the THC metabolites routinely found in the fatty tissue of human brains, reproductive organs, and other fatty areas of the body to the dead brain cells in the suffocated monkeys.
The government passed off the brain cell death rate contributed from carbon monoxide, ah THC in order to squash it.
In 1983 the Reagan Bush administration told universities and researchers to destroy all 1966-76 cannabis research work.
Now, in 1999, 17 years have passed and not a single word of Dr. Heath's or Dr. Nahas' research has been verified!
But their studies are still hauled out by the Partnership for a Drug Free America, the Drug Enforcement Administration, city and state narcotics bureaus, plus politicians and, in virtually all public instances, held up as scientific proof of the dangers of marijuana.
This is U.S. government propaganda and disinformation at its worst!
The public paid for these studies and has the right to the correct information and history being taught in our taxpayer sponsored schools.
In 1996, Gabriel Nahas, in France, sued Mishka, the translator of the French edition of this book, "L'Emperor est Nu!", for damages.
Mishka wrote that Nahas' studies were viewed by the world as garbage.
The French court, upon hearing all the testimony by Nahas, and after Nahas had spent an quivalent of tens of thousands of American dollars on legal fees, awarded him its highest insult: one franc, the equivalent of approximately 15 cents American for damages, and no legal fees!
We only found out about the Nixon, Marijuana, and the Shafer Commission study a few years ago thinks to dedicated medical & law professionals who filed freedom of rights of information request.
The government lied for many reasons, one of the main reason is pharmaceutical companies spend billions every year lobbying to keep cannabis illegal for the fact is they make trillions off cancer drugs & research.
they are already aware that cannabis cures cancer.
They have a grate con going at the moment.
Cancer patients & their loved ones will have to spend their entire life savings or even sell their houses & businesses in order to pay for chemotherapy & other cancer treatment drugs.
A lot of the time they spend & all that money spent, still their loved ones die anyway.
If the public found out that the government had been lying for over 40 years, that millions of lives could have been saved & the dying could grow the cure they needed in their own back yard the public would go ANARCHY.
Smoking cannabis dose not cure cancer, eating, juicing, or eating cannabis oil does.

What is the significance of U.S. government patent number 6 million 630,507.
The U.S. government has a patent on cannabis.
This official government document unequivocally states that cannabinoids are useful in the prevention and treatment of a wide variety of diseases including autoimmune disorders, Stroke, Trauma, Parkinson's, Alzheimer's, HIV, Dementia, cancer and Nausea,
The patent, awarded in 2003 is based on research done by the national institute of health and is assigned to the U.S. department of health and human services.
So! Why is this important?
here is a legal document in the public domain, which flies in the face of the U.S. government's stated position with regard to the classification of cannabis as a scheduled 1 substance having no currently excepted medical use.
Spread word of this to everyone you know.
Citing this pattern will stop the medical marijuana is a myth advocates dead in their tracts.
They simply cannot argue with it. The forces that will keep cannabis illegal are vocal and well funded, but they are not impervious to persistent effort.
The lynchpin in the war on drugs is cannabis.
Without the suppression and interdiction of this popular and widely used substance, have simply would not be enough for legal drug used going on to justify the huge amount of money and resources spent on fighting drugs.
I believe disseminating this information to as many people as possible is a crucial strategy in loosening the lynchpin's stranglehold in order to awaken public conscious perception on the fundamental importance of safe aguaculture, ecological, industrial hemp and medical cannabis.
The war on drugs is a failed and destructive policy, marijuana smokers are not a threat to society.
One day, the existence of this irrefutable patent will become widely held public knowledge and the government's rhetoric will be shown to be as hollow as a busted drum.
A world where alcohol is a perfectly legal substance attainable almost everywhere yet cannabis users are outlawed in present of their possessions stolen by the courts; quite simply: offense reason!

And Today Big
Pharma Are Making Billions....
Millions Are Dying From Their Drugs.... And The Rockefeller's.... Well They Are Part Of The New World Order (LUCIFERIANS ILLUMONATI)
Millions Are Dying In Plain Sight As Well Helps Their Population Reduction Plan.
Although David Rockefeller just passed away his families legacy of depopulation plan will carry on unless we do something about it.
you possess the power to impact the global narrative & to be part of the history

The Endocannabinoid System
Obesity, particularly abdominal obesity and the associated cardio metabolic complications are critical areas of investigation. The endocannabinoid system helps to regulate the central control of energy balance and peripheral metabolic processes, both of which may contribute to cardio metabolic risk factors ENDOCANNABINOID SYSTEM an overview. The endocannabinoid system or ECS influences multiple physiologic processes. This intricate system moderates energy intake as well as nutrients transport, metabolism and storage. The ECS regulates these processes through andodgelis ligands such as anandamide, 2 erekidonal glycerol and the CB1 receptor. CB1 receptors are located in the brain, digestive tract, muscle and adipose tissue. integration of these central and peripheral ECS components is achieved through neuronal and hormonal singling. ENDOCANNABINOID BIOLOGY Within the brain CB1 receptors are among the most abundant G protein coupled receptors, however in contrast to classical singling where information travails from pre to post
Synaptic neurons the ECS uses retrograde singling. The information travails from post to pre synaptic neuron. Let's take a closer look at this mechanism using a glutamate turgic neuron model. When in action protintural reaches the axiom terminal maim brain depolarization triggers the release of glutamate. Glutamate binds to post synaptic glutamate receptors inducing calcium channels to open. During periods of intense neural activity, calcium accumulates in the post synaptic neuron. This calcium buildup causes the synthesis and release of endocannabinoids from maim brain lipids. Diffusing across the synaptic cleft, the cannabinoids bind to the CB1 receptor activating the G protein. Activation influences ion flow. The result, suppression of pre synaptic neural transmitter release endocannabinoids are subsequently taken back into the cell and intermatically degraded. In addition to acting as neural messengers ENDOCANNABINOIDS mediate paracrine and autocuing signaling in adipocyte's podocyte's and other cells. ENDOCANNABINOID SYSTEM APPETITE, MOTIVATION AND SATIETY endocannabinoid system activity in the central nerves system regulates food intake. for example ECS stimulation by hunger and fasting singles, stimulates apatite and increases the palatability of food. endocannabinoids slow gastric emptying and GI transit and appear to stimulate secretion of ghrelin a neural peptide that increases apatite and food intake. After eating polosistic quinine in the droning triggers satiety signals. Subsequently ECS activity is decreased through suppression of CB1

expression. An increase in the adiposity hormone lepton decrease endocannabinoid levels in the athepatimos and decreases food intake. ENDOCANNABINOID SYSTEM PERIPHERAL METABOLIC REGULATION ECS regulation of peripheral metabolism influences energy balance. Stimulation of the ECS increases food intake and adiposity. Conversely blocking CB1 receptors reduces food intake and adiposity. In the liver ECS stimulation can lead to lipogenesis through the activation of hepatic lipogenic enzymes and increase fatty acid synthesis. ENDOCANNABINOID SYSTEM DYSLIPIDEMIA AND GLUCOSE HOMEOSTASIS Chronic stimulation of the ECS is associated with dyslipidemia. Activation of CB1 receptors increases expression of SREDP1C a lipogenic transcription factor and increase fatty acid synthesis. SREDP1C increases production of lipogenic enzymes ACC1 and fatty acid synthase. Increase fatty acid synthesis can lead to production of large triglyceride rich VLDL. Large triglyceride rich VLDL sets the stage for the atherogenic lipid profile of small dense LDL, decreased levels of apron protective HDL and overall increases in cholesterol and triglycerides levels. Adiponectin, another hormone secreted by adipocytes regulates lipid and glucose metabolism. Adiponectin is believed to regulate fatty acid oxidation in muscle and liver thus improving insulin sensitivity. CB1 receptor stimulation in adipocytes reduces Adiponectin, while CB1 blockade increases Adiponectin synthesis. Metabolic deregulation leads to a constellation of symptoms including abdominal obesity, atherogenic dyslipidemia, hypotension, insulin resistance, prothrombotic state and proinflammatory state. As basic and clinical research progresses we will continue to increase our understanding of the central and peripheral endocannabinoid system and it's roll in the regulation of metabolic function.

In the normal transmission of pain ascending nose septic singles travail up the spinal caloric pathway to the thalamus where they are processed and relayed to cortical and other areas. The ascending pathway also relays to the Periaqueductal gray matter, an important part of the descending pain modulating system where specific cannabinoid receptors are located. Stimulation of the Periaqueductal gray is known to produce an analgesic effect, however this neuroregulatory proses is thought to be tonically restricted by inhibitory gabaergic off cells. Ascending pain signals relay through the Periaqueductal gray suppress the activity of gabaergic off cells through cannabinoid modulation thereby increasing activity in the descending pain modulation pathway and increasing the analgesic effect. This is called depolarization induced suppression of inhibition. A synaptic feedback mechanism is believed to be an inherent part of our normal neuroregulatory processes Including descending pain modulation. In many regions of the CNS including the Periaqueductal gray the generation of postsynaptic action potentials is modified by an endogenous cannabinoid regulatory system. Endogenous cannabinoids are synthesize on demand from the phospholipid bilayer of the post synaptic midbrain. These lipophilic ligands are released directly into the synaptic cleft and act in retrograde fashion on the presynaptic neuron where the cannabinoid receptors are expressed. Binding of the endogenous cannabinoids effects intracellular signal transduction pathways reducing the influx of calcium ions into the presynaptic neuron causing a decrees in further neurotransmission release. This intern influences the frequency of post synaptic firing. The administration of exogenous cannabinoids orally work through inhalation may therefore provide an option for treating pain that has responded parley to conventional pain therapy. When exogenous cannabinoids are administered orally they travail veal the GI tract to the liver where they are metabolized before entering the blood stream and then the CNS. The onset of action from oral administration is less rapid compared to the inhaled route. When exogenous cannabinoids are inhaled they travail through the respiratory tract to the alveoli of the lung where they defuse rapidly into the circulatory system. They thin travail through the blood stream toured the CNS where they defuse across the blood brain barrier and into the surrounding tissues of the brain and spinal cord. Exogenous cannabinoids exert their modulatory effects upon the abundant cannabinoid receptors distributed throughout the CNS. The effects of cannabinoids may be illustrated by comparing three normal synapses showing normal pain transmission, the influences of endogenous cannabinoids and the influence of exogenous cannabinoid administration. Exogenous cannabinoids from the blood stream bind to cannabinoid receptors on the presynaptic neuron and mimic the endogenous synaptic modulatory effect by decreasing influx of calcium ions in this way pain and other neural processes are modulated. The clinical effects of exogenous cannabinoid administration are not limited to pain and have proven effects including the reduction of anxiety, spasticity, anorexia and nausea. The development of normal drugs to modulate endogenous cannabinoid reuptake in metabolism may also prove beneficial in pain therapy. The therapeutic benefits of cannabinoids including the modification of the endogenous cannabinoid singling system should be further explored through clinical research

The EC system consists of cannabinoid receptors, the endocannabinoids, and all the factors involved in the synthesis and degradation of endocannabinoids.
Pineal, Hypothalamus, Pituitary Thyroid, Parathyroids, Thymus, Adrenals, Pancreas, Ovary, Testes.
They claim it was established at least 600 million years ago. The EC system has a slightly different function in vertebrates and in invertebrates. A survey of amphibians and birds has shown that the ECB signaling acts as a kind of behavioral switch that allows the redirection of the less meaningful to the more urgent types of behavior in case of emergency adjustment to geoclimatic changes. The EC system has contributed significantly to the development of most living creatures as it began to develop simultaneously with the first living creatures, it's development is very diversified and interwoven with various animals and plant species.
Peripheral nervous system, cranial nerves, spinal nerves.
Cannabinoids are an essential element of the suction reflex in infants and are associated with digestive reflexes and appetite.

Pharynx, Oral Cavity, Tounge, Salivary, Glands, Parotid, Sublingual, Submandibular, Esophagus, Diaphragm, Liver Gallbladder, Stomach, Pancreas, Pancreatic Duct,
Common Blue Duct, Duodenum, Transverse Colon, Ascending Colon, Cecum, Appendix, Small Intestines, Descending Colon, Rectum, Anal Canal, Anus.
THC and endocannabinoids reduce the parasympathetic function and the vomiting reflex. Cannabinoids. Also protect the heart by reducing high blood pressure.
Aorta, Superior Vena Caval, Pulmonary Arteries, Inferior Vena Cava, Descending Aorta, Femoral Vein, Femoral Artery.
The EC system is a vital element in shaping the corticothalamic synaptic circuit in embryos.
Orbital Gyri, DM Cingulate, Gyrus, ATM Striatum, Frontal Gyri, Precentral Gyrus, Postcentral Gyrus, VN Temporal Gyri, Parietal Gyri, CGN, TRN, NGN.
The use of hemp promotes neurogenesis, the formation and growth of new brain cells in hippocampus, which is related to antianxiolitic, antidepressant effects and the feeling of euphoria. Hemp is the only external source of cannabinoids, hormones that are essential for the proper functioning of our body, Action Potential 2-AG e CB production Ca2 AMPA receptors and play a key role in embryonic development of the brain, the formation of brain cells and synapses.
Dopamine terminal, dopamine, dopamine receptors, THC, THC receptor terminal, THC receptors,
Concentrations of CB1 receptors
Cerebellum1 Movement, Basal Ganglia1 Movement, Cerebral Cortex1 Higher cognitive function, Hippocampus1 Learning, memory, stress, Spinal Cord1 Peripheral sensation including pain, Hypothalamus2 Appetite, Medulla3,4 Nausealvomiting, chemotherapy trigger zone (CTZ).
Formation of new brain cells in adults is crucial for all healing processes.
Cerebral Cortex, Pineal Gland, Suprachiasmatic Nucleus, Optic Chiasm, Pituitary, hypothalamus.
The cannabinoid receptors are condensed in the brain and spinal cord, but mostly in the hippocampus, amygdala, Cerebral Cortex, and pineal gland, Medial Orbito-Fruntal, Frontal Pole, Superior Frontal, Paracentral lobule, Precuneus, Cuneus, Lingual, Fusiform, Rostral Anterior Cingulate, Caudal Anterior Cingulate, Isthmus, Temporal Pole👈, Entorhinal, Parahippocampal, Pericalcarine
especially in the frame of cortico-talamic synaptic circuits responsible for awareness.
Parietal (Attentional Expression), Frontal (Attentional control) Thalamus.
The EC system is instrumental in shaping synaptic connections between the cerebral cortex and ganglia, which are responsible for targeted and reasonable behavior,
Basal Ganglia, Thalamus, Amygdala, Nucleus Accumbens,
VYA, Frontal Cortex, Ventral pallidum, Stratum, Substantia Nigra,
Cerebellum, Hippocampus, Brain Stem,
while disruption of these connections are the cause of many mental disorders. (Pineal Gland)
Cannabinoids are responsible for the transmission of visual stimuli from the pineal gland to the cortex, and also have an important role in recognizing and identifying visual impulses and pictures.
Cerebellum, Midbrain, Corpus Callosum, Thalamus,
Hypothalamus, Pituitary Gland Mammillary Body, Pons, Medulla oblongata, Spinal Cord,
hieroglyph, eye of Horus (Corpus Callosum, Thalamus, Medulla Oblongata Hypothalamus)
The EC system is controlled by an electrical frequency of the pineal gland (thalamus), which is essential to maintain the balanced functioning of an organism,
while disturbances in the electrical functioning of the pineal gland cause seizures. The EC system is involved in the functioning of the entire body.
Organs Of the Immune System.
Tonsils and adenoids, Lymph nodes, lymphatic vessels Thymus armpit Lymph Nodes Spleen,
Appendix, Peyer's Patches Lymph nodes in legs, Lymphatic Vessels, bone marrow.
One of the tasks of endocannabinoids is the defense system against excitotoxicity,
Viruses, Toxins, carcinogens Funguses Pollution, Bacteria, Germs, Parasites.
Disturbances in the EC system are reflected as different nerve diseases.
Jaka has noticed that there are similarities of the endocannabinoid and the biophotonic system. The biophotonic system is namely our electromagnetic field, consisting of photons, weak wave-particles of the visual spectrum. According to the energy theory, the photons are the building blocks of the universe, originating in time before there were any elements or atoms. Photons have a remarkable ability to connect and form frequency nets. Their communication is instant regardless of distances. Photons can also save information as memories. Large quantities of photons can be stored in crystals for unlimited time and activated later or transformed into another kind of energy. Both ECS and PBS, are concentrated mainly in the brain and spine cerebellum, Corpus callosum body of fornix, pituitary gland, especially in the thalamus and the cortex. Photonic therapy has much milder, though similar effect as cannabis therapy, namely antidepression and neurogenesis. While the ECS is responsible for the short term memory, the BPS is responsible for the long term memory. Jaka thinks it is no coincidence, that THC, CBD, CBN and all the other 85 known cannabinoids are concentrated in the hemp crystals, as photonic energy can concentrate in crystals and the ECS can in essence be viewed as energy transfer between the subatomic level and the molecular level.

Certain chemicals contained inside of the cannabis plant called cannabinoids might be the most powerful and non inhaled edible nutrients mankind will ever know. They may do fore medicine what germ theory and antibiotics did ushering in the next grate advancement in human health and life expectancy. Cannabis was used as a medicine for thousands of years and was even part of the US pharmacopeia from 1850 to 1942 at witch point it was removed from the American medical establishment and made illegal for all purposes based on races fears and claims that its recreational use gave black men the ability to seduce white woman, that weed caused insanity and schizophrenia, weed gave Mexicans super human straight turning them into blood thirsty violent murders and rapist, that weed led to pore choices such as breaking glass coke bottles open to drink resulting in swallowed glass, that marijuana is deadly resulting in death by smoking it, was a stepping stone leading to hard core drugs such as heroin. Now the new lies are the use and distribution

Of weed propitiates gang wars and terrorism. today cannabis is filed as a schedule one drug under the controlled substances act, a category under which it is legally defined as having absolutely zero point zero zero excepted medical use and a high potential for abuse. This being true despite the fact that eighteen states including Washington DC recognize cannabis as a medicine. The public has known about the incredible medicinal Qualities of cannabis for decades now and has thought to allow it nationally as a medicine. The united states federal government has been extremely firm in its position fighting lawsuit after lawsuit to keep cannabis in its highly restrictive category. The thing is the feds position on the matter explicitly proscribes smoking as the method delivery to the body which they claim to have zero medical value. Generally speaking burning a substance, destroying most of it and inhaling the smoke that's created isn't the most effective way to deliver nutrients to the body. Throughout time the feds have never publicly acknowledged the possibility of eating cannabis cannabinoids as medicine. What's interesting about the stubborn stance government leaders has that cannabis has zero medical value is that they actually hold a US patent on the use of cannabis cannabinoids for the treatment and prevention of ischemic age related inflammatory and autoimmune diseases. The feds have known about the disease fighting exogenous chemical composition of cannabis since 1974 when they discovered the exogenous cannabinoids inside of it to have cancer fighting properties in mice. The national cancer institute of federal agency tested four common cannabis cannabinoids with lung cancer and on lung cancer cells in test tubes the cannabinoids were administered orally and ingested by the mice three out of the four cannabinoids reduced the size of some tumors and slowed the progression of others leading to a longer survival time than the untreated mice. The test tube cancer cells were also impaired by the cannabinoids. At that time science had no explanation why cannabinoids could show to be anti cancerest. The pieces started to come together in 1988 when endogenous cannabinoid receptors or endocannabinoid receptors was found in the brain of a rat soon after it was discovered that endocannabinoid receptors are replete throughout the mammalian body. They are in every bodily system, brain, blood cells, internal organs, skin, and bones. These receptors are not unique only to mammals. Every living thing that has a back bone and a central nerves system has endocannabinoids receptors. In mammals these receptors have been found to play a kea role in many bodily functions. Two distinct types of cannabinoid receptors have been identified and a third type is expected to exist. the two distinct types of endocannabinoid receptors help control different parts of the body in different ways. the endocannabinoid receptors are in charge of turning up activity in certain parts of the body and turning it down in others. Acting as regulators to keep bodily functions in check. Then in 1992 it was discovered that the body actually makes its own form of cannabinoids called endocannabinoids to feed there receptors and to allow them to do there important over cite job eating foods with omega 3 fatty acids helps the production of endocannabinoids such as anandamide or 2-erekidonoyalglycerol 2-AG which are thin created on demand by the body to feed any receptor that needs stimulation. The endocannabinoid 2- arachidonoylglycerol (2-AG) is in mothers milk. Scents discovering that endocannabinoid receptors impact apon so many bodily functions, scientists have been determined to experiment with chemicals that effect them and explore for their potential medical use. There have only been five distinct types of endogenous endocannabinoids identified to have been made inside the body there have been 85 uneak exogenous exocannabinoids identified so far in cannabis. There are cannabinoids in some other plants like Echinacea, but no where near the quantity or potential medical quality of cannabis. Because the 85 known cannabis cannabinoids are illegal to proscribe and prohibitively difficult to research for medical use in America researchers instead have been forced to create artificial cannabis mocking drugs to attempt to stimulate the endocannabinoid receptors and manipulate their actions in a helpful way. Schematic representation of the endocannabinoid system at a synaptic cleft. Endogenous cannabinoids are synthesized from membrane lipids in postsynaptic neurons after calcium (Ca++) increase . They diffuse and activate pre-synaptic CB1 receptors that indicate intercellular cascades, inhibiting neural activity and neurotransmitter release. Anandamide (arachidonoylethanolamide, AEA) undergoes re-uptake and hydrolysis by fatty acid amide hydrolase (FAAH) whereas 2-arachidonoyl glycerol (2AG) is broken down by monoacylglycerol lipase (MAGL). The existence of a membrane endocannabinoid-transporter (T) is controversial. The technical definition of a cannabinoid is any chemical that is capable of binding to and activating an endocannabinoid receptor. Cannabinoids made inside the body are called Endo (5+) endocannabinoids cannabinoids found in plants are called phyto(110+) cannabinoids and the rest, Artificial (17+) are made by scientists in a laboratory. These man made cannabinoids don't suffer the same automatic illegal statist as their natural counterparts. Section 812. Schedules of Controlled Substances (a) Establishment. There are established five schedules of Controlled substances, to be known as schedules 1, 2, 3, 4, and 5. Such schedules shall initially consist of the substance listed in this section. The schedules established by this section shell be updated and republished on a semiannual basis during the two-year pried beginning one year after October 27, 1970, and shall be updated and republished on an annual basis thereafter. (b) Placement on schedules; findings required Except where control is required by United States obligations under an international treaty, convention or protocol, in effect on October 27, 1970, and expect in the case of an immediate precursor, a drug or other substance may not be placed in any schedule unless the findings required for such schedules are made with respect to such drug or other substance. The findings required for each of the schedules are as follows

(1) Schedule

1.– (A) the drug or other substance has a high potential for abuse.

(B) the drug or other substance has no currently accepted medical use in treatment in the United States.

(C) There is a lack of accepted safety for use of the drug or other substance under medical supervision.

Synthetic cannabinoids that gain FDA approval are allowed to be marketed and soled on the highly expensive pharmaceutical market. Today there are two such artificial cannabinoids that can legally be prescribed to Americans. In 1985 the drug merijuanal was approved by the FDA and then entered the market. It is proscribed to reduce nausea and vomiting in chemotherapy patients and increase appetite in aids and chemo patients. Also in 1985 the drug Cesamet was approved by the FDA but it was not allowed to enter the market until 2006 it is proscribed to reduce nausea and vomiting and neuropathic pain in chemo patients and increase appetite in aids patients. Both merijuanal and Cesamet are modeled after the cannabis cannabinoid delta-9-tetrahydrocannabinol THC the only other pharmaceutical cannabinoid drug in the world is the low concentration oral spray Sativex. It interred the market in 2003 as a treatment for multiple sclerosis patents to alleviate their spasticity and neuropathic pain. Because Sativex contains the real cannabis cannabinoids THC and CBD it is illegal in America but available in other countries like Candia, England, Spain, and New Zealand. Although merijuanal and Cesamet are the extent to which an American can legally used cannabinoids the research shows endocannabinoid receptors could be used to do more than just fight symptoms. A search on PubMed.gov for the word cannabinoid yields 15243 result. These studies most of which involved non smoked cannabinoids show that endocannabinoid receptors

Could be utilized to have a uniquely profound impact on what is currently known as health. Schematic representation of endocannabinoids action. Endocannabinoids are synthesised in and released from the membrane of post-synaptic neurons on-demand after calcium influx (1).

They activate pre-synaptic CB1 receptors and restrain neural activity

(2) Anandamide and 2- arachidonoylethanolamide (2AG) are removed from the synaptic cleft by up-take into the post- and pre-synapse, respectively.

(3). Once inside neurons, anandamide binds TRPV1 (with consequences opposite to those of CB1 activation) and undergoes hydrolysis by FAAH, whereas 2-arachidonoylethanolamide, (2AG) is hydrolysed by MGL

This is no where near a complete list but here are 25 studies showing different diseases and conditions that could be treated or even healed by

exploiting the endocannabinoid system
breast Cancer artificial cannabinoid used in order to reduce the size and spreading of tumors
"Mice treated with JWH-133 or WIN -55,212-2 (artificial cannabinoids) showed a 40% to 50% reduction in tumor growth and a 65% to 80% reduction in lung metastasis."
Prostate Cancer an artificial cannabinoid reduced tumor growth
"WIN-55,212-2 (mixed CB1/CB2 agonist) (artificial cannabinoid) treatment…resulted in a dose- and time dependent inhibition of cell growth."
Lung Cancer artificial cannabinoids stop the growth and spreading of tumors
"Both CB1 and CB2 agonists Win55,212-2 and JWH-133, respectively, (artificial cannabinoid) significantly inhibited in vivo tumor growth and lung metastasis (50%)"
Pancreas Cancer artificial cannabinoid inhibited tumor growth
"(artificial cannabinoid) treatment strongly inhibits growth of human pancreatic tumor cells xenografted in nude mice without appearing toxic effects."
Liver Cancer THC and an artificial cannabinoid impaired cancer cells
"We found that THC and JWH-015 (an artificial cannabinoid) reduced the viability of human hepatocellular liver carcinoma cell line and hepatocellular carcinoma cells"
Cervical Cancer an artificial cannabinoid killed cancer cells
"MA- (artificial cannabinoid) induced apoptosis was confirmed in another human cervical cancer cell line"
Colon Cancer an artificial cannabinoid reduced cell growth and killed cancer cells
"Rimonabant (an artificial cannabinoid) significantly reduced cell growth and induced cell death"
Non-Hodgkin Lymphoma an artificial cannabinoid reduced tumor size
"In vivo treatment with R(+)-MA (an artificial cannabinoid) caused a significant reduction of tumor size"
Leukemia Delta-9-Tetrahydrocannabinol THC reduced tumor size and killed cancer cells
"Treatment of EL-4 tumor-bearing mice with Delta-9 tetrahydrocannabinol THC in vivo led to a significant reduction in tumor load, increase in tumor-cell apoptosis, and increase in survival of tumor-bearing mice"
skin cancer artificial cannabinoid reduced the growth of tumors
"Local administration of the mixed CB(1)/CB(2) agonist WIN-55,212-2 or the selective CB(2) agonist JWH-133 (artificial cannabinoid) induced a considerable growth inhibition of malignant tumors"
Cirrhosis an artificial cannabinoid reduced fibrosis
"fibrosis was significantly reduced in rats treated with Rimonabant (an artificial cannabinoid) compared with rats receiving the vehicle."
Hepatitis Delta-9-Tetrahydrocannabinol THC inhibited hepatitis
"Intraperitoneal administration of THC… inhibited hepatitis as shown by significant decrease in liver enzymes and reduced liver tissue injury."
Alzheimer's an artificial cannabinoid protected neurons and reduced cognitive impairment
"CB1 Agonist ACEA (an artificial cannabinoid) protects Neurons and Reduces the cognitive impairment of…mice."
Parkinson's THCV could slow progression of PD
"THCV has a promising pharmaceutical profile for delaying disease progression in PD"
Multiple Sclerosis artificial cannabinoid reduced neurological disability and progression of the disease
"the treatment of mice experimental brain inflammation with the cannabinoid agonist WIN55,12-2 (an artificial cannabinoid) reduced their neurological disability and the progression of the disease."
Arthritis artificial cannabinoids protected cartilage from damage and degradation
"It is concluded that biologically stable synthetic cannabinoid protect cartilage matrix from degradation induced by cytokines"
Cardiac Arrhythmia Anandamide and an artificial cannabinoid prevented arrhythmias
"It has been found that prior intravenous administration of the endocannabinoid anandamide (10 mg/kg) or it's synthetic analogue R-(+)-methanadamide (5mg/kg) prevents a development of ischemic and reperfusion arrhymias in rats
Insomnia an endocannabinoid increased sleep
"Anandamide (an endocannabinoid) increased adenosine levels in the basal forebrain and also increased sleep."
Sleep Apnea cannabinoids suppressed sleep related apnea
"This study demonstrated potent suppression of sleep-related apnea by both exogenous and endogenous cannabinoid."
Inflammatory bowel Disease artificial cannabinoids reduced inflammation
"Inflammation was significantly reduced in the presence of UBR597, VDM11, or both (artificial cannabinoids)"
Depression Endocannabinoids may treat depression and anxiety
"Animal experiments suggest that drugs promoting endocannabinoid action may represent a novel strategy for the treatment of depression and anxiety disorders."
Post Traumatic Stress disorder an artificial cannabinoid could help control nightmares
"The results of this study indicate the potential benefits of abialone, a synthetic cannabinoid, in patients with PTSD experiencing poor control of nightmares with standard pharmacotherapy."
HIV cannabinoids inhibited TAT
"Cannabinoids inhibit migration of microglial-like cells to the HIV protein TAT."
Osteoporosis an artificial cannabinoid increased bone mass and protected against bone loss
"Pharmacological and genetic inactivation of CB1,
CB2 and e GPR55 in adult mice suppress bone resorption, increase bone loss"
Nicotine Addiction an artificial cannabinoid could help smokers quit smoking
"These findings indicate significant, but limited, utility of e rimonabant (an artificial cannabinoid) for smoking cessation."
The bulk of trials that do moved on to humans are those that show potential that control symptoms. For some reason almost no experiments that show potential to actually cure disease move on to humans interestingly enough in all of history there hasn't been one study done testing the curative effect of ingesting cannabis cannabinoids on humans. The only study on humans testing the disease fighting properties of non smoked cannabis cannabinoids took place in Spain in 2006 they took the lone cannabis cannabinoid chemical THC and directly injected it into malignant brain tumors of patients. As suspected it had a cancer fighting reaction in the patients tumors which allowed them to live much longer than expected. Since then no more clinical trials of this nature have been preformed on humans. Despite the mountains of research showing the disease fighting exogenous plant based chemical cannabinoid composition potential of cannabis, both artificial cannabinoids available to sick Americans are modeled after the cannabis chemical THC and all they do is alleviate a few symptoms in 2007 using a new method to target the endocannabinoid receptors a French pharmaceutical company Sanofi Aventis who's logo is because health matters, came close to bringing America its next cannabinoid drug. They theorized that because CB1 receptors stimulate appetite, shutting those receptors down could help reduce appetite and help people lose weight. They didn't know exactly which CB1 receptors controlled appetite, so they made a drug that simple blocked as many CB1 receptors as possible. Their drug called Acomplia made it all the way to a FDA faze three trial patient were indeed showing signs of reduced appetite. The drug also showed potential uses for nicotine Addiction and metabolic disorders but the FDA stopped the trial. Some patients formed extreme anxiety and depression and attempted suicide. Patients with a history of depression weren't allowed in the study although Acomplia was not approved in the US it was approved in Europe and it was available in 56 countries by 2008. it crinkly became clear however that its negative effects out wade it's positive effects and it was polled from the market by the end of 2008. other pharmaceutical companies like American Pfizer were in reproduction stages with their own versions of weight loss CB1 receptor blockers. These programs were not fully carried out because of their failure of Acomplia. What Acomplia did is

show clearly that if at lease certain CB1 receptors did not receive endocannabinoids then the body could not function normally. Plain and simple cannabinoid receptors are part of the body and they need to be fed. In todays world do to one or a combination of certain factors such as age, weight, diet, lack of diet, free radicals, and environmental
Toxins, fore some people there could be a problem some where in the line of producing, transporting, and receiving all of the known endocannabinoids needed to keep their Endocannabinoid System receptors happy. Thus directly impacting proper function in specific parts of the body. A lack of Vitamin A leads to Exophthalmia-night blindness, a lack of Vitamin B12 leads to Megaloblastic Anemia-Subacute Combined Degeneration of Spinal Cord, a lack of Vitamin C leads to Scurvy, a lack of Vitamin D leads to Rickets, a lack of Vitamin K leads to Coagulation, a lack of Thiamine leads to Beriberi, a lack of Niacin leads to Pellagra, a lack of Endocannabinoids leads to depression. Over time this could lead to a condition similar to avitaminosis. Avitaminosis is any disease caused by a chronic long term prolonged Vitamin deficiency its already known that suppurating serten CB1 receptors for a short time increases the chances of depression. Considering the multitudes of bodily functions the CB1 and CB2 receptors control as a whole, who knows how many other conditions could be caused by a lack of nourishment in the receptors. Vitamins were only discovered because of the realization that if people eat certain plants they didn't get serten disease. The cure for the disease was as simple as eating those plants containing nutrients nature intended for the body. And as long as people got those nutrients regularly then disease would never develop. The chemicals in cannabis meaning both hemp and marijuana when they are fresh raw and unheated are not psychoactive. THCV contained only in living cannabis can't get you high. That means as long as you eat or juice it in its natural state cannabis is just another vegetable most modern pharmaceutical drugs focus only on a small concentrated part of the body and work to shut it down. it's possible that when ingested properly at least one of the 85 natural cannabis cannabinoid could safely fill the role of the endocannabinoids around the entire body taping into a natural built in system to help restore normal order to arias of the body that need it in a way that has never been intentionally utilized in all of known human history. The government will not let cannabinoids be officially fully tested as in the prevention or cure of disease, thus not allowing scientists to know the full potential of cannabinoid therapy in the roll as medicine or its nutritional value in the health and betterment of human kind. their are a lot of people out there who are claiming that ingested cannabis cannabinoids have cured their illnesses but their accounts are only anecdotal. Rather ingesting Cannabis cannabinoids fights deficiency in the body, provides an incredible boost to the health, or dose nothing at all we deserve to know. If you care about the millions of sick, the injustice, the out of control cost of health, care and the underfunded medicaid programs then position against the prohibition of natural therapies which are all being made illegal. The only way the government will ever do anything is if enough people speak out. The world can't afford to ignore cannabis any longer.

It's been used by human bings since the dawn of time. Early mans diet undoubtedly included cannabis since it grew politically in Africa and Asia. Pot is highly nourishing do to its protein, fiber, 8 amino fatty acid, omega 3 content. Marijuana is speculated to be one of the first cultivated plants. In resent years the hemp plant has been proven to be anti-spasmodic, anti-inflammatory and anti-bacterial. It can treat depression, dramatic stress syndrome, chronic pain, glaucoma, migraines, multiple sclerosis, turrets, nausea and a host of other elements without the unwanted side effects of pharmaceutical drugs. Historical evidence showed that the holy anointed oil used by old testament Hebrews contained cannabis extract. The early Christians used cannabis as part of there baptismal process. In the eighteen hundreds medical journals published more than a hundred articles on the therapeutic use of the drug then known as cannabis indica or indene hemp. Cannabis was often prescribed by doctors and was part of every medical bag right through the early part of the twentieth century. It was a popular treatment from labor pains, asthma eremitism and nerves disorders. It was even given to cranky babies to get them to stop crying. Then the dark days for pot began in the early part of the twentieth century with an advent of chemically created drugs and an emerging profit driven assembly line medical system. The NEW ORDER didn't want competition. Cannabis was still preferred by many patients and doctors to the new miracle drugs which were composed mainly of unnatural toxic chemicals. After lobbing by the pharmaceutical industry and fear mongering misinformation by there donation dependent allies in congress, a federal law passed in nineteen thirty seven that prohibited doctors from proscribing pot in any circumstances with threat of prosecution. Even though it was strongly apposed by the American medical association of its day all cannabis products were removed from the us pharmacal burial national formulary by nineteen forty two. After alcohol was relegalized, rather than go out of business the massive bureaucracy of prohibition turned its operation to marijuana eradication. This war on cannabis lasted until just a few years ago when states started passing their own medical marijuana initiatives in order to once again provide pot safely to patients. California was the first U.S. state to pass a medical marijuana law in nineteen ninety-six. The intent was to give relief to patients suffering from chronic pain, aides cancer and other illnesses. Sense nineteen ninety-six thirteen other American states have passed similar compassionate use laws, twelve more had ballet measures or legislation pending. Although cannabis smoke has been shown to have precancerous effect in animal tissue, countless study's have felled to find a link between cannabis smoking and cancer. In fact heavy cannabis users were shown to have lower cancer risks than the general population. Is it possible that the tar in smoked pot could be causing malignancies while the chemical constituents in pot are curing them at the same time? Although there's some concern that marijuana can exasperate schizophrenia and psychosis in a few patients already suffering from these diseases resent extensive studies in the U.K. have failed to hold support a causable relationship between cannabis and mental disorders. It was only in the 1960's that fat salable THC was isolated from pot plants and declared the substance that got us high. Cannabis can effect memory and movement but only temporarily. Though there have been reports of pots negative effects on the hart and

cardiovascular system, they are ware and not scientifically substantiated. Research has also shown that pass claims of brain damage from pot failed to hold up under scientific scrutiny. All in all cannabis is vary low risk. The long history of the human use of cannabis also attest to its safety. Nearly five thousand years without a single documented death. Medical marijuana dispensaries across America are now offering an oppressive array of cannabis enhanced foods which makes it easier to over medicate with marijuana edibles. they are neither toxic nor lethal and the symptoms will were off in a few hours. For marijuana to prove fatal you'll have to smoke fifteen hundred pounds within fifteen minutes and any resulting death would likely be from asphyxiation. Doosan's of major studies have been passed in the last few years that indicate that chemicals in cannabis in the lab and in animals have a significant effect in fighting almost all major cancers including brain, breast, prostate, lung, thyroid, colon, skin, pituitary, melanoma, and leukemia cancers. They do this by promoting the death of cancer cells that have forgotten how to die as well as a reduction in there crucial blood supply while leaving healthy cells untouched. But why you may wonder how would cannabis have any effect on cancer. The answer can be explained in one word. Endocannabinoids. Amazing as it sounds were all born with a form of cannabis already in our bodies. It's called the endocannabinoid system. The endocannabinoid system or ECS influences multi physiological processes. This intercut system moderates energy intake as well as nutrient transport, metabolism and storage. A completely natural collection of compounds, endocannabinoids are our bodies form of marijuana and are involved in most of our cells and structures. They control a variety of functions in the nerves system, heart, reproductive and immune systems. Endocannabinoid messengers help the cells communicate. Typically they protect our good cells while killing the bad ones like cancer cells. In all animals the nerves system is made of the same components. Large numbers of nerve cells carrying electrical signals and where ever the cells meet these signals are passed to a receptor in the next cell by a chemical messenger called a neural transmitter. Inside the brain there are different types of neural transmitters including dopamine and serotonin. All life with a nerves system is integrated with this basic structure. Because of these similarities these new signals came to be known as cannabinoids. It was inevitable that cannabis would eventually meet its perfect partner. Humanity. Rather you like it or not each and every one of us is fundamentally wired to respond to cannabis. After scientist discovered THC, they found that it could binned to specific receptors in the brain and the area around it and that this interaction created a cascade of biological processes leading to the well known high. If you block the endocannabinoids artificially in mice they tend to grow tumors and they also get depressed. A few years ago some brilliant entrepreneurs came up with the idea that blocking the endocannabinoids in our body to create a new diet drug. The theory being if cannabis gives people the munchies, then blocking the endocannabinoids would make them lose their apatite's. The drug they developed was Rimonabant which did indeed decrease apatite by blocking the endocannabinoid receptors, but data from clinical trials showed that Rimonabant users suffered depression, anxiety, insomnia and digressive impulses at trice the rate of subjects given a placebo. In one study there were five suicides among Rimonabant users because as they discovered, endocannabinoids are also mood regulators with the capacity to make us feel euphoric or when blocked depressed. Rimonabant was finally withdrawn from the market in two thousand eight researchers at the MD Anderson cancer center in Texas reported that mice on Rimonabant developed Particularly cancerous plops at a far higher rate than controls confirming that endocannabinoids are not only mood regulators but tumor regulators as well. What's especially fascinating is that while significant numbers of endocannabinoids are not found in breast and prostate cells, the homes for the two leading causes of cancer it appears that large quantities show up only after normal cells begin to progress toward a state of uncontrolled growth in a desperate attempted to restrain the tumorest invaders. The endocannabinoids already inside our bodies did an efficient job for thousands of years working as tumor regulators, free radical scavengers and anti-inflammatory agents. Then once we entered the industrial age with all it's pollution and chemical indoctrine of food, the endocannabinoids became overwhelmed with the task of keeping us healthy and protected from toxins. Better public health helped us live longer but unfortunately human binges became innovated with the free radicals our bodies produced naturally by simply being alive along with an unprecedented Tyrannical valiance in doctrine of GMO's carcinogens and contaminates. Including pharmaceutical drugs. The endocannabinoids needed help. ENTERED CANNABIS! When marijuana is consumed and broken down naturally into a collection of cannabinoid molecules the THC and its sisters seek out and binned with the endocannabinoid receptors already inside us. Together they give the body more ammunition agents all of the diseases of modern times. Scientist are now experimenting to see witch parts of the pot plant including its cannabinoids and other biological compounds might be the most effective agents a variety of diseases including diabetes and cancer. Despite all the difficulties there's a group of doctors in the U.S. and abroad who have been advancing the research on the medical use of cannabis with highlly impressive and sometimes revolutionary discoveries. Seth research laboratories in California recently demonstrated that in some tumors cancer cells are killed by marijuana while the other healthy cells are left untouched cells that stop moving and become still white dots are dead cancer cells. The ability of cannabinoids to kill bad cells while protecting healthy ones is Particularly impotent when we are talking about brain cancer because of the blood brain barrier. The brain has to be sheltered from outside influences that might hitch a ride in the blood stream and cause havoc. What is exiting and unique about cannabinoids is that they can pass through the blood brain barrier because of their slippery fat loving nature. Cannabinoids get right into the brain's cancer cells by moving easily through the cells midbrain which are also composed of lipids. The evidence is piling up in mice infested labs at the endocannabinoid system when stimulated by cannabinoids has an anti tumor effect and can instruct cancer cells to commit suicide. Doctor Seminaire Manuel Guzman is a professor of biochemistry and molecular biology in Madrid Spain and is known for his ground breaking studies on medical cannabis. If cannabis might be the miracle cancer cure everybody's been searching for then why don't doctors everywhere know about it. Why aren’t billions of funds being directed towered cannabinoid research by the organization that raze money for cancer therapies. It's only an aromatic herb yet almost every government in the world restricts or bands Its use. In many countries cannabis suppliers and users can still be put in jail for life or even executed. Why dose the corporate media continue to connect marijuana only with gangs, Violent Drug dealers, race, societies losers and not the growing number of doctors and scientist who are discovering its benefits. It is true that for those who use it regularly pot can change your way of thinking and can make people question why they should continue to do things that aren’t making them happy. In essence grass can make you feel that the past and the future are not as important as the present. It has the potential to altar the vary nature of consciousness by opening up the mind to unpredictable possibilities. That doesn't sit well with those who insists on remaining in control. Cannabis is a natural substance therefor couldn't be patented until the twentieth century. Anyone with a green thumb can grow it. So its production could be in the hands of the people instead of the corporations that run a two hundred billion dollar a year cancer business. It may not be a coincidence that pharmaceutical company are silently preparing for what could easily become a total revolution in the curing habits of the worlds population. G.W. pharmaceuticals a company that specializes in the study and commercialization of cannabinoids has already established a full cycle facility in undisclosed location in the south of England where they oversee the cultivation of the plant right to the finished medical product. This facility grows thirty to sixty thousand plants every year once the marijuana is ready for processing it undergoes the same exact treatment, extraction and refinement that is forbidden to regular citizens around the world. Cannabis contains four hundred and twenty-one chemicals. 85 chemical compounds from cannabis are cannabinoids. Cannabinoids are a unique class of compounds not found anywhere else in nature. It's a colorful plant with antioxidant activity. Cannabis is subject to few predators and has even been utilized as an insect repellent. Another stress to which pot plants are subject results from their daily exposure to the radiation produced by ultraviolet sunlight. This has created certain Unique resistant defenses in cannabis to ultraviolet U.V U.V.A. U.V.P. solar radiation. The same chemical that protects the plant may well have a preventive anti-cancer effect inside all animal and human cellular reparative structures. Since cannabinoids are scavengers of disease causing free radicals. There are four resistant compounds that are of the most interesting to scientists doing medical research. Delta-9 tetrahydrocannabinol or THC is the principal psychoactive compounded ingredient that creates euphoria when smoked, vaporized or ingested once after it has deride out. THC exerts a wide spectrum of biological effects by mimicking the bodies own natural endocannabinoids. Faced with this rabidly changing scenario the drug enforcement administration is trying to modify it's official position to allow pharmaceutical companies to market THC without appearing to contradict all the negative information on Marijuana they have been propagating for decades. The DEA now recognizes the medical qualities of THC by trying to shift the blame from the prohibition of marijuana to the possible damage caused by the smoke. Cannabidiol, also known as CBD is a non psychoactive component of cannabis that gives the sedative effect to the high experienced amongst its numerous functions. CBD has been shown to have anti-inflammatory analgesic effects. It helps fight diabetes, bacterial infections, and malignant tumors. This compound is also a nerve protectant with well documented anti-psychotic and anxiety reducing effects. Tetrahydrocannabivarin, or THCV has been found in significant amounts mostly in south African and some Thai strains of grass. High concentrations of THCV will make the high come on quicker but last for less time. This chemical compound may eventually prove to be a useful treatment for type two diabetes. THCV has been shown to have a protective, preventative

effect against malignant tumors. Cannabichromene, or CBC is a low level cannabinoid though little research has been done scientist believe CBC may have an antidepressant effect as well as the ability to reduce inflammation. CBC strongly inhibits tumor growth in leukemia and breast cancer. Faced with all these new discoveries, one is brought to question what stops marijuana from being legalized for medical use all around the world It's not only doctors calling for legalization. After becoming a perfect fixer in the urban panorama in California Oregon is beginning to open the first public venues for medical marijuana smokers. At the federal level they are still clinging to a more archaic view and equate arkaim with cannabis To discourage church goers from using it under any circumstances. The anticancer qualities of marijuana appear to have been known to the US government for over forty years. In a nineteen seventy four seventy five medical collage of Virginia study, led by doctor Albert Monson discovered that THC slowed the growth of three kinds of cancer, Lung, breast and virus induced leukemia. The Virginia study was shout down and all further cannabis tumor research was abandoned What is a weed? A plant whose virtues have not been discovered. Ralph Waldo Emerson.

How Cannabis Oil Works to Kill Cancer Cells

Bio-chemist Dennis Hill
By: Dennis Hill

First let’s look at what keeps cancer cells alive, then we will come back and examine how the cannabinoids CBD (cannabidiol) and THC (tetrahydrocannabinol) unravels cancer’s aliveness.

In every cell there is a family of interconvertible sphingolipids that specifically manage the life and death of that cell. This profile of factors is called the “Sphingolipid Rheostat.” If endogenous ceramide(a signaling metabolite of sphingosine-1-phosphate) is high, then cell death (apoptosis) is imminent. If ceramide is low, the cell is strong in its vitality.

Very simply, when THC connects to the CB1 or CB2 cannabinoid receptor site on the cancer cell, it causes an increase in ceramide synthesis which drives cell death. A normal healthy cell does not produce ceramide in the presence of THC, thus is not affected by the cannabinoid.

The cancer cell dies, not because of cytotoxic chemicals, but because of a tiny little shift in the mitochondria. Within most cells there is a cell nucleus, numerous mitochondria (hundreds to thousands), and various other organelles in the cytoplasm. The purpose of the mitochondria is to produce energy (ATP) for cell use. As ceramide starts to accumulate, turning up the Sphingolipid Rheostat, it increases the mitochondrial membrane pore permeability to cytochrome c, a critical protein in energy synthesis. Cytochrome c is pushed out of the mitochondria, killing the source of energy for the cell.

Ceramide also causes genotoxic stress in the cancer cell nucleus generating a protein called p53, whose job it is to disrupt calcium metabolism in the mitochondria. If this weren’t enough, ceramide disrupts the cellular lysosome, the cell’s digestive system that provides nutrients for all cell functions. Ceramide, and other sphingolipids, actively inhibit pro-survival pathways in the cell leaving no possibility at all of cancer cell survival.

The key to this process is the accumulation of ceramide in the system. This means taking therapeutic amounts of CBD and THC, steadily, over a period of time, keeping metabolic pressure on this cancer cell death pathway.

How did this pathway come to be? Why is it that the body can take a simple plant enzyme and use it for profound healing in many different physiological systems? This endocannabinoid system exist in all animal life, just waiting for its matched exocannabinoid activator. This is interesting. Our own endocannabinoid system covers all cells and nerves; it is the messenger of information flowing between our immune system and the central nervous system (CNS). It is responsible for neuroprotection, and micro-manages the immune system. This is the primary control system that maintains homeostasis; our well being.

Just out of curiosity, how does the work get done at the cellular level, and where does the body make the endocannabinoids? Here we see that endocannabinoids have their origin in nerve cells right at the synapse. When the body is compromised through illness or injury it calls insistently to the endocannabinoid system and directs the immune system to bring healing. If these homeostatic systems are weakened, it should be no surprise that exocannabinoids are therapeutic. It helps the body in the most natural way possible.

To see how this works we visualize the cannabinoid as a three dimensional molecule, where one part of the molecule is configured to fit the nerve or immune cell receptor site just like a key in a lock. There are at least two types of cannabinoid receptor sites, CB1 (CNS) and CB2 (immune). In

general CB1 activates the CNS messaging system, and CB2 activates the immune system, but it's much more complex than this. Both THC and anandamide activate both receptor sites. Other cannabinoids activate one or the other receptor sites. Among the strains of Cannabis, C. sativa tends toward the CB1 receptor, and C. indica tends toward CB2. So sativa is more neuroactive, and indica is more immunoactive. Another factor here is that sativa is dominated by THC cannabinoids, and indica is predominately CBD (cannabidiol).

It is known that THC and CBD are biomimetic to anandamide, that is, the body can use both interchangeably. Thus, when stress, injury, or illness demand more from endogenous anandamide than can be produced by the body, its mimetic exocannabinoids are activated. If the stress is transitory, then the treatment can be transitory. If the demand is sustained, such as in cancer, then treatment needs to provide sustained pressure of the modulating agent on the homeostatic systems.

Typically CBD gravitates to the densely packed CB2 receptors in the spleen, home to the body's immune system. From there, immune cells seek out and destroy cancer cells. Interestingly, it has been shown that THC and CBD cannabinoids have the ability to kill cancer cells directly without going through immune intermediaries. THC and CBD hijack the lipoxygenase pathway to directly inhibit tumor growth. As a side note, it has been discovered that CBD inhibits anandamide reuptake. Here we see that cannabidiol helps the body preserve its own natural endocannabinoid by inhibiting the enzyme that breaks down anandamide.

This brief survey touches lightly on a few essential concepts. Mostly I would like to leave you with an appreciation that nature has designed the perfect medicine that fits exactly with our own immune system of receptors and signaling metabolites to provide rapid and complete immune response for systemic integrity and metabolic homeostasis.
- See more at: http://www.cureyourowncancer.org/how-cannabis-oil-works.html#sthash.9YssPpV6.dpuf

From <http://www.cureyourowncancer.org/how-cannabis-oil-works.html>

THE ENDOCANNABINOID SYSTEM: PHYSIOLOGY AND PHARMACOLOGY

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Abstract

The endogenous cannabinoid system is an ubiquitous lipid signalling system that appeared early in evolution and which has important regulatory functions throughout the body in all vertebrates. The main endocannabinoids (endogenous cannabis-like substances) are small molecules derived from arachidonic acid, anandamide (arachidonoylethanolamide) and 2-arachidonoylglycerol. They bind to a family of G-protein-coupled receptors, of which the cannabinoid CB1 receptor is densely distributed in areas of the brain related to motor control, cognition, emotional responses, motivated behaviour and homeostasis. Outside the brain, the endocannabinoid system is one of the crucial modulators of the autonomic nervous system, the immune system and microcirculation. Endocannabinoids are released upon demand from lipid precursors in a receptor-dependent manner and serve as retrograde signalling messengers in GABAergic and glutamatergic synapses, as well as modulators of postsynaptic transmission, interacting with other neurotransmitters, including dopamine. Endocannabinoids are transported into cells by a specific uptake system and degraded by two well-characterized enzymes, the fatty acid amide hydrolase and the monoacylglycerol lipase. Recent pharmacological advances have led to the synthesis of cannabinoid receptor agonists and antagonists, anandamide uptake blockers and potent, selective inhibitors of endocannabinoid degradation. These new tools have enabled the study of the physiological roles played by the endocannabinoids and have opened up new strategies in the treatment of pain, obesity, neurological diseases including multiple sclerosis, emotional disturbances such as anxiety and other psychiatric disorders including drug addiction. Recent advances have specifically linked the endogenous cannabinoid system to alcoholism, and cannabinoid receptor antagonism now emerges as a promising therapeutic alternative for alcohol dependence and relapse.

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INTRODUCTION

Twenty-four years of pharmacological research separate the identification of the main psychoactive constituent of Cannabis sativa preparations, (−)-Δ9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964; Mechoulam, 1970) from the characterization (Devane et al., 1988; Herkenham et al., 1991) and molecular cloning (Matsuda et al., 1990) of its cellular target, the cannabinoid CB1 receptor (CB1). The extensive research on the structure and activity of the natural constituents of Cannabis (termed cannabinoids) and the development of synthetic compounds with high potency and stereoselectivity have led to the identification of the main physiological functions that are modulated by this new class of drugs (Howlett et al., 1990). The discovery of the cannabinoid receptor and the availability of highly selective and potent cannabimimetics led to the rapid identification of a family of lipid transmitters that serve as natural ligands for the CB1 receptor: arachidonoylethanolamide (AEA), named anandamide from the

Sanskrit ‘internal bliss’ (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). The pharmacological properties of the endocannabinoids were found to be very similar to those of the synthetic cannabimimetics. The subsequent description of a complex biochemical pathway for the synthesis, release (Di Marzo et al., 1994; Cadas et al., 1996), transport (Beltramo et al., 1997) and degradation (Cravatt et al., 1996) of endocannabinoids completed the scaffold of a new signalling system termed the ‘endocannabinoid system’. Since the discovery of anandamide, more than 3500 scientific reports have comprehensively explored the main aspects of the endocannabinoid system. This system now appears as a relevant modulator of physiological functions not only in the central nervous system but also in the autonomic nervous system, the endocrine network, the immune system, the gastrointestinal tract, the reproductive system and in microcirculation (Di Marzo et al., 1998; Table 1).

Enlarge table

Table 1.

A selection of reviews and reports that explore in depth the main aspects of endocannabinoids and their receptors

Topic	References
Biochemistry and molecular biology	Matsuda, 1997; Felder and Glass, 1998; Giuffrida et al., 2001; Piomelli, 2003
Signalling	Schlicker and Kathmann, 2001; Wilson and Nicoll, 2002; Freund et al., 2003
Anatomy and development	Breivogel and Childers, 1998; Fernandez-Ruiz et al., 2000; Elphick and Egertova, 2001
Physiology	Di Marzo et al., 1998; De Petrocellis et al., 2004
Pharmacology	Howlett et al., 2002
Addiction	Maldonado, 2002; Maldonado and Rodríguez de Fonseca, 2002; Tanda and Goldberg, 2003; Martin et al., 2004
Pain	Calignano et al., 2000; Pertwee, 2001
Behaviour	Rodríguez de Fonseca et al., 1998; Chaperon and Thiebot, 1999; Castellano et al., 2003
Gastrointestinal system	Izzo et al., 2001
Immune system	Cabral, 2001
Cardiovascular system	Kunos et al., 2002; Randall et al., 2002; Hiley and Ford, 2004
Therapeutic applications	Piomelli et al., 2000; Cravatt and Lichtman, 2003; Guzman, 2003; Smith et al., 2004

Since the discovery of anandamide in 1992, over 3500 publications have reported new data on the biological role of the endogenous cannabinoid system.

The present review gives a general perspective of the endogenous cannabinoid system, including the main pharmacological advances in the development of drugs capable of modulating their dynamics. The review focuses on the role of endocannabinoids as modulators of reward circuits and motivated behaviour that are relevant for drug addiction, including alcoholism. In light of the extensive research over the past 12 years, several specialized reviews wherein the reader will find a more profound analysis of the role played by the endocannabinoid system in selected physiological functions are shown in Table 1.

Biochemistry of the endogenous cannabinoid system

Endocannabinoids. When discovered, the endocannabinoids were found to be derivatives of arachidonic acid, which resembled other lipid transmitters (eicosanoids such as prostaglandins or leukotrienes). Additional studies revealed the existence of other structure-related lipid messengers including palmitylethanolamide or oleoylethanolamide, which are not active at cannabinoid receptors. These messengers will not be included in this review, although they serve important physiological functions in inflammation, pain control, feeding behaviour and lipid metabolism (Calignano et al. 1998; Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Piomelli, 2003).

Endocannabinoids are derivatives of arachidonic acid conjugated with ethanolamine or glycerol. Figure 1 depicts the chemical structure of four endocannabinoids, anandamide, 2-arachidonoylglycerol (2-AG), the ester of arachidonic acid and ethanolamine; virodhamine which resembles anandamide (Porter et al., 2002), and the 2-arachidonyl glyceryl ether noladin, an analogue of 2-AG (Hanus et al., 2001). All these endocannabinoids have been found in the brain, plasma and peripheral tissues, although the relevance of noladin has been questioned recently (Oka et al., 2003) because its concentration in the brain is too low for this compound to act as an endogenous cannabinoid receptor ligand. In the brain, the concentration of anandamide is 200-fold lower than that of 2-AG (Sugiura et al., 1995; Stella et al., 1997). The monoglyceride 2-AG is a metabolic intermediate in lipid metabolism whereas anandamide is the product of the cleavage of a membrane phospholipid. However, after depolarization or receptor stimulation (e.g. dopamine D2 receptor-mediated), the concentration of anandamide can rise up to 5–12 fold in a time-limited fashion (Giuffrida et al., 1999; Stella and Piomelli, 2001; Kim et al., 2002).

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Fig. 1.

Cannabinoid receptor agonists. Left, the structure of four arachidonic acid derivatives that have been identified as endogenous ligands for both the cannabinoid CB1 and CB2 receptors. Right, the structure of Δ9-tetrahydrocannabinol (THC), the main cannabinoid receptor agonist present in Cannabis preparations and that of the aminoalkylindole WIN-55,2122, a synthetic cannabinoid receptor agonist active at CB1 and CB2 receptors.

Synthesis and release. Different pathways are involved in the synthesis and release of anandamide and 2-AG. Figure 2 shows the dynamics of formation and degradation of anandamide.

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Fig. 2.

Overview of the biochemical pathways for synthesis, degradation and cellular actions of the endogenous cannabinoid anandamide. Anandamide is released from a membrane lipid precursor (N-arachidonoyl-phosphatidylethanolamine, NAPE) by the action of a specific phospholipase D (PLD) activated by depolarization or G-protein-coupled receptor (GPCR) stimulation. NAPE biosynthesis is catalysed by a membrane enzyme, N-acyltransferase (NAT) activated by calcium (Ca2+) and cAMP. Anandamide acts as a retrograde messenger at presynaptic cannabinoid receptors (CB1), where it regulates neurotransmitter release (NT) through its second transduction systems [mainly Ca2+ incorporated through voltage-gated calcium channels (VGCC) or glutamate NMDA (N-methyl-D-aspartate) receptors]. Anandamide also acts as a neuromodulator of major transmitter systems, including dopamine, at postsynaptic cells, where it regulates excitability and synaptic plasticity through its modulation of potassium (K+) channels, and the regulation of a broad spectrum of protein kinases (PK) including protein kinase A and mitogen-activated protein kinases (MAPK). Anandamide action is terminated through a two-step process, which includes, first, its cellular uptake through a specific anandamide transporter (AT) and second, degradation by enzymatic cleavage to arachidonic acid (AA) and ethanolamide by the membrane-bound enzyme fatty acid amidohydrolase (FAAH).

Anandamide is formed by the cleavage of a phospholipid precursor, the N-arachidonoyl-phosphatidylethanolamine (NAPE). The precursor is synthesized by the enzyme N-acyltransferase (NAT), which catalyses the transfer of arachidonic acid from phosphatidylcholine to the head group of phosphatidylethanolamine. This enzyme requires the presence of Ca2+ and is regulated by cAMP, which enhances the activity of NAT by phosphorylation mediated through the cAMP-dependent activity of protein kinase A (Cadas et al., 1996; Piomelli, 2003). The release of anandamide from NAPE is catalysed by a specific phospholipase D (PLD), which has been cloned recently (Okamoto et al., 2004). This enzyme has no homology with the known PLD enzymes and is classified as a member of the zinc metallohydrolase family. Its presence is highest in the brain, kidneys and testis. The activity of PLD is regulated by depolarization or by activation of the ionotropic glutamate N-methyl-D-Aspartate (NMDA) receptors or nicotinic α7 neuronal receptors (Stella and Piomelli, 2001; Piomelli, 2003) or stimulation of the metabotropic receptors of major neurotransmitters including dopamine, glutamate and acetylcholine (Giuffrida et al., 1999; Varma et al., 2001; Kim et al., 2002).

The synthesis and release of 2-AG is different from that of anandamide. Because 2-AG is a monoglyceride, its formation is closely associated with the metabolism of triacylglycerol, mainly by the receptor-dependent activation of phosphatidylinositol-specific phospholipase C (PLC). The standard

model proposes that activation of metabotropic receptors coupled to the PLC and diacylglycerol (DG) lipase pathway will systematically lead to increases in 2-AG production (Stella et al., 1997; Piomelli, 2003). Cloning of the enzyme 1,2-diacylglycerol lipase (Bisogno et al., 2003) has confirmed this hypothesis, as well as the contribution of ionotropic purinergic receptors such as P2XT, which boosts 2-AG formation (Witting et al., 2004). Although 2-AG formation is dependent on Ca2+, its regulation is independent of anandamide synthesis and release. Once anandamide and 2-AG are formed, they target the CB1 receptors in the same cell where they were formed, via diffusion within the plasmalemma, or they can be released to the extracellular fluid where they reach distant targets (i.e. presynaptic terminals) with the apparent help of protein carriers such as lipocalins or albumin (Piomelli, 2003).

Uptake and degradation. Endocannabinoid signalling is terminated by a two-step process that includes transport into cells and hydrolysis by two specific enzymatic systems. Both steps exert a tight control of endocannabinoid levels in tissues, rapidly eliminating these signalling molecules. Endocannabinoid uptake is mediated by a transporter (Beltramo et al., 1997), which is widely distributed throughout the brain (Giuffrida et al., 2001). The transporter is an elusive molecule which works in a manner that is similar to other lipid carriers: it facilitates the uptake of both anandamide and 2-AG in an energy-independent fashion (Beltramo et al., 1997). The anandamide transporter is saturable, displays substrate specificity and can be blocked by specific drugs such as AM 404 (Fig. 4). A major issue of debate has been the potential coupling of endocannabinoid transport and degradation: it is possible that the energy for the uptake process is obtained by its coupling to the enzymatic hydrolysis of anandamide. However, a recent report seems to confirm that transport and degradation are independent processes (Fegley et al., 2004). The degradation of endocannabinoids is performed by two specific enzymatic systems: the fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) and the monoacylglyceride lipase (MAGL) (Dinh et al., 2002). FAAH is a membrane enzyme that belongs to the serine–hydrolase family. FAAH is widely distributed throughout the body, with high concentrations in the brain and liver. FAAH can degrade many fatty acid amides, including acylethanolamides such as anandamide and the sleep factor oleamide. Although FAAH can inactivate 2-AG, the main enzyme responsible for the inactivation of this monoglyceride is MAGL (Dinh et al., 2002). This enzyme is also a serine hydrolase and its distribution in the nerve terminals of specific brain neurons has been determined recently (Gulyas et al., 2004).

Receptors. Two major cannabinoid receptors have been cloned, both of which belong to the superfamily of G-protein-coupled receptors. The first receptor described was named the CB1 receptor and it is mainly located in the terminals of nerve cells (central and peripheral neurons and glial cells), the reproductive system (i.e. testis), some glandular systems and the microcirculation (Devane et al., 1988; Howlett et al., 1990; Herkenham et al., 1991; Wagner et al., 1997; Batkai et al., 2001). The CB2 cannabinoid receptor was found initially in multiple lymphoid organs with the highest expression detected in B lymphocytes, moderate expression in monocytes and polymorphonuclear neutrophils and the lowest expression in T lymphocytes, although subsequent studies identified it in microglial cells as well (Munro et al., 1993; Galiègue et al., 1995; Piomelli, 2003). An interesting aspect of cannabinoid receptors is their expression during development of the brain, where they control cell differentiation (Rueda et al., 2002), and their presence in tumour cells derived from glial cells and the main epithelia (Galve-Roperh et al., 2000; Sanchez et al., 2001; Casanova et al., 2003). Pharmacological studies revealed the existence of other endocannabinoid targets including the vanilloid receptor (Zygmunt et al., 1999) and at least two non-CB1 non-CB2 ‘CB-like’ receptors, one in the vascular bed and the other in glutamatergic axon terminals (Hajos et al., 2001; Howlett et al., 2002; Kunos et al., 2002). The existence of these and other putative cannabinoid receptors, and their role in endocannabinoid physiology can be clarified only after their molecular characterization. Cannabinoid receptors, especially the CB1 receptor, display unique properties. The most relevant property is their preservation throughout evolution: e.g. human, rat and mouse CB1 receptors have 97–99% amino acid sequence identity. The preservation of this ancient signalling system in vertebrates and several invertebrate phyla reflects the important functions played by the endocannabinoids in cell and system physiology. A second remarkable characteristic of the CB1 receptors is their high expression in the brain. The CB1 receptor is the most abundant G-protein-coupled receptor, with densities 10–50 fold above those of classical transmitters such as dopamine or opioid receptors (Howlett et al., 1990; Herkenham et al., 1991). Another important characteristic is the low efficiency of CB1 receptor coupling to its transduction system: e.g. when compared with opioid receptors, CB1 receptors are 7-fold less efficient in their ability to couple to G proteins (Breivogel et al., 1998; Felder and Glass, 1998; Manzanares et al., 1999).

Both cannabinoid receptors are coupled to similar transduction systems. Cannabinoid receptor activation was initially reported to inhibit cAMP formation through its coupling to Gi proteins (Devane et al., 1988; Howlett et al., 1990), resulting in a decrease of the protein kinase A-dependent phosphorylation processes as well. However, additional studies found that the cannabinoid receptors were also coupled to ion channels through the Golf protein, resulting in the inhibition of Ca2+ influx through N (Mackie and Hille, 1992), P/Q (Twitchell et al., 1997) and L (Gebremedhin et al., 1999) type calcium channels, as well as the activation of inwardly rectifying potassium conductance and A currents (Mackie et al., 1995; Childers and Deadwyler, 1996). These actions are relevant to the role of cannabinoids as modulators of neurotransmitter release (Schlicker and Kathmann, 2001) and short-term synaptic plasticity (Wilson and Nicoll, 2001), as discussed below. Further research also described the coupling of CB1 and CB2 receptors to the mitogen-activated protein kinase cascade, to the phosphatidylinositol 3-kinase, to the focal adhesion kinase, to ceramide signalling and to nitric oxide production (Derkinderen et al., 1996; Bouaboula et al., 1997; Molina-Holgado et al., 1997; Galve-Roperh, 2000; Howlett et al., 2002). Finally, recent studies revealed that under certain conditions, the CB1 receptors can stimulate formation of cAMP by coupling to the Gs protein (Felder et al., 1998).

Endocannabinoids exhibit different binding properties and intrinsic activity at CB1 and CB2 receptors. Anandamide behaves as a partial agonist at both CB1 and CB2 receptors, but has higher affinity for the CB1 receptor (Hillard et al., 1999; Howlett et al., 2002). The intrinsic activity of anandamide at CB1 receptors is 4–30 fold higher than at CB2 receptors. However, 2-AG is a complete agonist at both CB1 and CB2 receptors and it exhibits less affinity than anandamide for both CB1 and CB2 receptors (Stella et al., 1997; Howlett et al., 2002).

Functional neuroanatomy of the endogenous cannabinoid system

As described above, the endogenous cannabinoid system is widely distributed throughout the body. In the peripheral tissues the localization of the elements of the endogenous cannabinoid system reflects the distribution of the cell types where they are located (e.g. B lymphocytes in spleen and lymph nodes). However, in the nervous system the distribution is much more complex and structured, and clearly reflects the importance of this system in synaptic transmission. In some regions, such as the hippocampus, there is a complementary distribution of cannabinoid receptors, endocannabinoid transporters and degradation enzymes. However, in other areas of the brain, for instance the thalamus, there are discrepancies (i.e. transport activity and MAGL expression in the absence of a relevant presence of the CB1 receptors) in its distribution, which reflects the gaps in our knowledge of the composition of the endocannabinoid system.

Receptors. From the early work of Herkenham et al. (1991) it was clear that the CB1 receptor distribution was unique among G-protein-coupled receptors, not only because of the very high densities of cannabinoid binding sites but also because of the dynamics of CB1 receptor synthesis and transport. Binding studies and in situ hybridization analysis showed that the cannabinoid receptors are synthesized in somata and the protein transported to axon terminals (Herkenham et al., 1991; Matsuda et al., 1993). The phenotype of the CB1 receptor-expressing neurons corresponds mainly to GABAergic neurons including cholecystokinin-containing neocortical, amygdalar and hippocampal neurons and dynorphin- and substance P-expressing medium spiny neurons of the outflow nuclei of basal ganglia (Tsou et al., 1999; Julian et al., 2003). Several glutamatergic and cholinergic telencephalic and cerebellar neurons also express the CB1 receptors (Piomelli, 2003). In the peripheral nervous system, the CB1 receptors are located in sensory neurons of the dorsal root ganglia. Figure 3 shows how the CB1 receptors are synthesized in medium spiny neurons of the caudate-putamen and the protein transported to the axon terminals in the globus pallidus and substantia nigra. The dense presence of CB1 binding sites in the cerebellum, hippocampus, striatum, globus pallidum and substantia nigra clearly reflects this biological characteristic of CB1 receptors.

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Fig. 3.

Imaging cannabinoid CB1 receptor in circuits of the rat brain reward system. Cannabinoid receptors are mainly located at presynaptic axon terminals. In the basal ganglia, CB1 receptor mRNA expression (panels A and B) is located mainly in GABAergic projecting neurons of the caudate-putamen (CPu), but not in the target nuclei, the globus pallidus or the substantia nigra (GP and SN). However, the protein is mainly detected by

immunohistochemistry (panels C and D) in the axon terminals innervating both outflow nuclei of the basal ganglia. Panel E shows the dense presence of CB1 receptors in the substantia nigra and ventral tegmental area (VTA) as mapped by CB1 receptor agonist-stimulated GTP-γ-S incorporation. In these areas, CB1 receptors are not located in dopaminergic neurons (Panel F): confocal imaging using specific antibodies against CB1 receptors (green) and tyrosine hydroxilase (red) shows the compartmentalization of CB1 receptors in GABAergic afferents to the substantia nigra pars reticulata (SNr), whereas dopaminergic cells are restricted to the pars compacta (SNc). The segregation of CB1 receptors and catecholaminergic transmission is also observed in the hippocampus-dentate gyrus (Hpc-DG, panel G).

Enzymes. Fatty acid amide hydrolase is present in large principal neurons, such as the pyramidal cells of the cerebral cortex, the pyramidal cells of the hippocampus, the Purkinje cells of the cerebellar cortex and the mitral cells of the olfactory bulb.

Immunocytochemical analysis of these brain regions revealed a complementary pattern of FAAH and CB1 expression with CB1 immunoreactivity occurring in fibres surrounding FAAH-immunoreactive cell bodies and/or dendrites (Egertova et al., 2003). This complementary distribution suggests that FAAH closely controls the duration of cannabinoid effects, although there are sites where this association does not occur, such as the outflow nuclei of basal ganglia. Monoglyceride lipase is located mainly in the hippocampus, cortex, cerebellum and anterior thalamus, with moderate expression in the extended amygdala, including the shell of the nucleus accumbens (Dinh et al., 2002). Comparison of the distribution of FAAH and MAGL at the cellular level shows that FAAH is primarily a postsynaptic enzyme, whereas MAGL is presynaptic. The spatial segregation of the two enzymes suggests that anandamide and 2-AG signalling may subserve functional roles that also involve spatial segregation, raising a controversy with respect to the nature and function of the retrograde endocannabinoid signal (Gulyas et al., 2004).

Transporter. The distribution of the anandamide transporter has been only partially characterized because the transporter has not been cloned. The distribution of transport activity is highest in areas expressing CB1 receptors, such as the hippocampus, the amygdala, the striatum and the somatosensory, motor and limbic areas of the cortex. Transport activity is also present in areas with low expression of the CB1 receptor, such as the thalamus and the hypothalamus (Beltramo et al., 1997; Giuffrida et al., 2001).

Pharmacology of the endogenous cannabinoid system

During the last twenty years, and especially after the discovery of the CB1 receptor and anandamide, an intense research effort has yielded numerous series of drugs that interact with most of the main elements of the endogenous cannabinoid system. Today we have drugs that bind to the CB1 receptor as agonists or antagonists, drugs that block the endocannabinoid transport and drugs that inhibit the activity of FAAH. We lack specific NAT, PLD, sn1-DAGL and MAGL inhibitors. Both in vitro and in vivo bioassays have been used to evaluate the activity of the new compounds.

Prior to the availability of radioligand cannabinoid receptors, in vitro assays included the inhibition of forskolin-stimulated cAMP production and the inhibition of electrically evoked contractions of isolated smooth muscle preparations. Smooth muscle preparations most often used for the bioassay of cannabinoids are the mouse-isolated vas deferens and the myenteric plexus-longitudinal muscle preparation from the guinea pig small intestine. These bioassays, which are particularly sensitive, rely on the ability of cannabinoid receptor agonists to act via the CB1 receptors to inhibit electrically evoked contractions. In vivo bioassays include behavioural tests for analgesia and locomotion. A cluster of four effects (analgesia, hypothermia, immobility and catalepsy) in mice constituting the ‘mouse tetrad’, is classically considered as a signature of cannabimimetic activity. The recent availability of mouse knockouts for the cannabinoid receptors and FAAH (Ledent et al., 1999; Cravatt et al., 2001) has facilitated these studies, offering a reliable model in the search for selective compounds.

What is the logic of a cannabinoid approach to pharmacotherapeutics? Cannabinoid receptor agonists may be designed to mimic the signalling processes mediated by anandamide and 2-AG, mainly in pathological situations where a boost in cannabinoid receptor stimulation might be needed. Cannabinoid receptor antagonism might be the approach selected in conditions with enhanced endocannabinoid signalling. Transport inhibition and inhibition of degradation are more sophisticated approaches, both oriented towards magnifying the tonic actions of endocannabinoids. A rational use of these therapeutic strategies requires the identification and evaluation of the functional status of endocannabinoid signalling in reference disorders. Thus, a deficit of anandamide signalling during conditions of stress might be counteracted by the blockade of anandamide degradation (Kathuria et al., 2003).

As a summary of cannabinoid pharmacology, Table 2 shows the reference compound for each molecular target, indicating Ki in the case of ligand–receptor interaction or IC50 in the case of enzymatic inhibitors.

Enlarge table

Table 2.

Targeting the endogenous cannabinoid system: synthetic drugs of reference for cannabinoid CB1 and CB2 receptors, anandamide transporter (AT) and endocannabinoid degradation enzyme, fatty acid amidohydrolase (FAAH)

Name	Target	Action	Ki/IC50 (nM)	Reference
ACEA	CB1	Agonist	1.4	Hillard et al., 1999
SR141716A	CB1	Antagonist	5.6	Rinaldi-Carmona et al., 1994
HU-308	CB2	Agonist	22.7	Hanus et al., 1999
SR 144528	CB2	Antagonist	0.60	Rinaldi-Carmona et al., 1998
UCM 707	AT	Blocker	800	Lopez-Rodriguez et al., 2001
OL-135	FAAH	Inhibitor (reversible)	2.1	Lichtman et al., 2004
URB 597	FAAH	Inhibitor (irreversible)	4.6	Kathuria et al., 2003

Cannabinoid receptor agonists. According to the International Union of Pharmacology (reviewed in Howlett et al., 2002), cannabinoid agonists can be divided into classical cannabinoids, non-classical cannabinoids, aminoalkylindoles and eicosanoids. New series of compounds have been recently described, including diarylether sulfonylesters (Mauler et al., 2002) and pyrrole derivatives (Tarzia et al., 2003b).

Classical cannabinoids are tricyclic dibenzopyran derivatives that are either compounds occurring naturally in the plant C. sativa, or synthetic analogues of these compounds. The most representative forms are Δ9-THC (Fig. 1), a partial agonist at both the CB1 and CB2 receptors and the main psychoactive constituent of Cannabis, along with 11-hydroxy-Δ8-THC-dimethylheptyl (HU-210), a synthetic compound that displays the highest potency at the CB1 receptor (Howlett et al., 2002). Classical cannabinoids are usually CB1/CB2 agonists, although changes in the THC molecule have led to the synthesis of selective CB2 receptor agonists such as HU-308 (Hanus et al., 1999).

Non-classical cannabinoids are synthetic THC analogues that lack the dihydropyran ring. The most representative form is the Pfizer compound CP-55 940, a potent and complete agonist at both the CB1 and CB2 receptors, which was used to characterize the CB1 receptor for the first time (Devane et al., 1988; Herkenham et al., 1991).

Aminoalkylindoles were the first non-cannabinoid molecules that displayed cannabimimetic activity (Pacheco et al., 1991).

R-(+)-WIN-55,212–2 (Fig. 1) is the most representative form, and it behaves as a complete agonist at both the CB1 and CB2 receptors, with higher intrinsic activity at the CB2 receptor.

Eicosanoids are the prototypic endocannabinoids (Fig. 1), of which anandamide (a partial agonist at both the cannabinoid receptors) and 2-AG (a complete agonist at both the CB1 and CB2 receptors) are the most representative compounds. Based on the structure of anandamide, minor chemical changes have led to the development of the first generation of CB1-selective agonists, of which R(+)-methanandamide and arachidonyl-2′-chloroethylamide (ACEA) (Table 2) are the most representative forms (Hillard forms et al., 1999).

Cannabinoid receptor antagonists. Several series of compounds have been developed as CB1 receptor antagonists. The most representative are diarylpyrazoles, substituted benzofuranes, aminoalkylindoles and triazole derivatives.

Diarylpyrazoles include both the first CB1 receptor antagonist synthesized (SR 141716A, Rinaldi-Carmona et al., 1994) and the first CB2 receptor antagonist (SR 144528). They were synthesized by Sanofi and are considered the reference antagonists. However, they are not neutral antagonists since they display significant inverse agonist properties. Modification of the SR 141716A molecule has yielded other CB1 receptor antagonists with improved properties, including SR 147778 and AM 281 (Howlett et al., 2002; Rinaldi-Carmona et al., 2004). Diarylpyrazoles are orally active and are currently under clinical trials for the treatment of obesity.

Substituted benzofuranes include LY 320135, a CB1 receptor antagonist with affinity at serotonin and muscarinic receptors (Felder et al., 1998).
Aminoalkylindoles include a CB2 receptor antagonist, AM 630, which also displays activity as a low-affinity partial CB1 agonist (Howlett et al., 2002).
Triazole derivatives include LH-21 (Jagerovic et al., 2004), an in vivo CB1 antagonist with a paradoxic low affinity in vitro for CB1 receptors and devoid of inverse agonist properties.
Uptake blockers. Based on the structure of anandamide, a series of eicosanoid derivatives that have the ability to block anandamide transport have been synthesized. The molecular structures of the three prototypical uptake blockers are depicted in Fig. 4. The first and best studied transport inhibitor is AM 404 (Beltramo et al., 1997). The administration of AM 404 results in the accumulation of anandamide and potentiates the effects of exogenously administered anandamide. The compound AM 404 can be degraded by FAAH and behaves as an agonist of vanilloid receptors. A second series of compounds is represented by UCM 707, which displays a higher affinity at the transporter than AM 404 (Lopez-Rodraíguez et al., 2001; De Lago et al., 2002). A latest addition is AM 1172, a FAAH-resistant transport inhibitor that allows the study of anandamide uptake processes without interference in FAAH activity (Fegley et al., 2004). However the IC50 of AM 1172 (2000 nM) is lower than that reported for UCM 707 (800 nM).

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Fig. 4.
Structure of three anandamide uptake blockers. UCM 707 is the compound with the highest affinity at the anandamide transporter. AM 404 was the first blocker designed and has been extensively described. Both molecules, however, had a significant impact on the activity of the fatty acid amidohydrolase (FAAH), the enzyme that degrades anandamide. AM 1172 is a recently described compound without inhibitory action at FAAH, which has been used to demonstrate the independence of anandamide transport and degradation processes.
Inhibitors of fatty acid amide hydrolase. As in the case of the cannabinoid receptors, different lines of research have led to the discovery of chemically heterogeneous FAAH inhibitors. The earlier inhibitors described consisted of reversible electrophilic carbonyl inhibitors (trifluoromethyl ketones, alpha-keto esters and amides, and aldehydes) or irreversible inhibitors (sulfonyl fluorides and fluorophosphonates) incorporated into the fatty acid structures. Based on the structure of alpha-trifluoromethyl ketones a series of potent inhibitors were developed. Of these, alpha-keto N4-oxazolopyridine provides inhibitors that are 102–103 times more potent than the corresponding trifluoromethyl ketones (Boger et al., 2000). A recent series of alpha heterocycles has been shown to possess very high potency and selectivity to reversibly inhibit FAAH activity in vivo and in vitro. The most potent of these new compounds is OL-135, which exhibits IC50 in the low nanomolar range (Lichtman et al., 2004). A different strategy has been selected by the group of Piomelli et al., who have developed exceptionally potent irreversible FAAH inhibitors, which exhibit a promising anxiolytic profile (Kathuria et al., 2003; Tarzia et al., 2003a). These new classes of inhibitors are carbamate derivatives capable of directly interacting with the serine nucleophile of FAAH. However, these new inhibitors, although extremely potent, are not selective because they may potentially inactivate other serine hydrolases such as heart triacylglycerol hydrolase (Lichtman et al., 2004).
Physiology of the endogenous cannabinoid system
The ubiquitous presence of the endogenous cannabinoid system correlates with its role as a modulator of multiple physiological processes. A comprehensive analysis of all the functions of the endocannabinoids is beyond the scope of the present review. The reader will find an extensive list of recent reviews that explore the physiological relevance of the endogenous cannabinoid system, as depicted in Table 1. In this section, we focus on the cellular and system physiological events mediated by endocannabinoids that are relevant to our understanding of the contribution of the endogenous cannabinoid system in alcoholism.
Cellular physiology. As described in the section on biochemistry of the endogenous cannabinoid system, endocannabinoids are released upon demand after cellular depolarization or receptor stimulation in a calcium-dependent manner. Once produced, they act on the cannabinoid receptors located in the cells surrounding the site of production. This property indicates that endocannabinoids are local mediators similar to the autacoids (e.g. prostaglandins). In the CNS, the highly organized distribution of endocannabinoid signalling elements in GABAergic and glutamatergic synapses and their preservation throughout evolution suggests a pivotal role in synaptic transmission. Because of the inhibitory effects on adenlyl cyclase, the activation of K+ currents and the inhibition of Ca2+ entry into cells, the net effect of the CB1 receptor stimulation is a local hyperpolarization that leads to the general inhibitory effects described. If endocannabinoids act postsynaptically they will counteract the activatory inputs entering the postsynaptic cells. This mechanism has been proposed for postsynaptic interactions with dopaminergic transmission (Felder et al., 1998; Rodríguez de Fonseca et al., 1998; Giuffrida et al., 1999). Despite its importance, this effect is secondary to the important presynaptic actions whose existence is supported by two facts: (i) the concentration of the CB1 receptors in presynaptic terminals and (ii) the well-documented inhibitory effects of the CB1 receptor agonists on the release of GABA, glutamate, acetylcholine and noradrenaline (Schlicker and Kathmann, 2001; Piomelli, 2003). This inhibitory effect has been demonstrated for neuropeptides such as corticotrophin-releasing factor and cholecystokinin as well (Rodríguez de Fonseca et al., 1997; Beinfeld and Connolly, 2001). Presynaptic inhibition of neurotransmitter release is associated with the inhibitory action of endocannabinoids on Ca2+ presynaptic calcium channels via the activation of CB1 receptors. Presynaptic inhibition of transmitter release by endocannabinoids may adopt two different forms of short-term synaptic plasticity, depending on the involvement of GABA or glutamate transmission, respectively: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) (Wilson and Nicoll, 2002; Diana and Marty, 2004). Both forms of synaptic plasticity involve the initial activation of a postsynaptic large projecting neuron (pyramidal or Purkinje cells) that sends a retrograde messenger to a presynaptic GABA terminal (DSI) or a presynaptic glutamate terminal (DSE), inducing a transient suppression of either the presynaptic inhibitory or the presynaptic excitatory input. The contribution of endocannabinoids to these forms of short-term synaptic plasticity has been described in the hippocampus (Wilson and Nicoll, 2001; Wilson et al., 2001) and the cerebellum (Diana et al., 2002). The nature of the endocannabinoid system acting as a retrograde messenger is still unknown. The role of endocannabinoid-induced DSI or DSE seems to be the coordination of neural networks within the hippocampus and the cerebellum that are involved in relevant physiological processes, such as memory or motor coordination.
Additional forms of endocannabinoid modulation of synaptic transmission involve the induction of long-term synaptic plasticity, namely long-term potentiation (LTP) and long-term depression (LTD). Both forms of synaptic plasticity involve long-term changes in the efficacy of synaptic transmission in glutamatergic neurons, which have a major impact on consolidation and remodelling of the synapsis. Activation of the cannabinoid receptors prevents the induction of LTP in the hippocampal synapses (Stella et al., 1997) and a facilitation of LTD in the striatum (Gerdeman et al., 2002) and the nucleus accumbens (Robbe et al., 2002). In the hippocampus, the endocannabinoid messengers regulate a form of LTD that affects inhibitory GABAergic neurons (Chevaleyre and Castillo, 2003).
Overall, endocannabinoids act as local messengers that adjust synaptic weight and contribute significantly to the elimination of information flow through specific synapses in a wide range of time frames. The fact that cannabinoid receptor stimulation has a major impact on second messengers involved not only in synaptic remodelling (Derkinderen et al., 1996; Piomelli, 2003) but also in neuronal differentiation (Rueda et al., 2002) and neuronal survival (Panikashvili et al., 2001; Marsicano et al., 2003) indicates that the signalling system is a major homeostatic mechanism that guarantees a fine adjustment of information processing in the brain and provides counterregulatory mechanisms aimed at preserving the structure and function of major brain circuits. Both processes are relevant for homeostatic behaviour such as motivated behaviour (feeding, reproduction, relaxation, sleep) and emotions, as well as for cognition, since learning and memory require dynamic functional and morphologic changes in brain circuits. An experimental confirmation of this hypothetical role of the endogenous cannabinoid system was the demonstration of its role in the control of the extinction of aversive memories (Marsicano et al., 2002; Terranova et al., 1996).
System physiology. The cellular effects of endogenous cannabinoids have a profound impact on the main physiological systems that control body functions (Table 1). Despite the peripheral modulation of the immune system, vascular beds, reproductive organs, gastrointestinal motility and metabolism, the endogenous cannabinoid system tightly regulates perception processes including nociception (cannabinoids are potent analgetics, Martin and Litchman, 1998) and visual processing in the retina (Straiker et al., 1999). Additional functions exerted by the endogenous cannabinoid

system involve the regulation of basal ganglia and cerebellar circuits, where it is involved in the modulation of implicit learning of motor routines (Rodriguez de Fonseca et al., 1998).

Among the varied functions in which the endogenous cannabinoid system is engaged, the homeostatic control of emotions and the regulation of motivated behaviour merit special attention because of its impact on human diseases, including addiction. The endogenous cannabinoid system controls the motivation for appetite stimuli, including food and drugs (Di Marzo et al., 1998, 2001; Navarro et al., 2001; Gomez et al., 2002). The positive effects of endocannabinoids on motivation seem to be mediated not only by the peripheral sensory systems in which cannabinoid receptors are present (i.e. the promotion of feeding induced by cannabinoid CB1 receptor agonists, Gomez et al., 2002), but also by the action of endocannabinoids on the reward system, a set of in-series circuits that link the brain stem, the extended amygdala and the frontal executive cortex. The endogenous cannabinoid system is widely distributed in the extended amygdala, a set of telencephalic nuclei located in medial septal neurons, the nucleus accumbens shell and amygdalar complex, and are involved in the control of motivated behaviour, conditioned responses and gating-associated emotional responses. This hypothesis is supported by two facts: the inhibition of motivated behaviour observed after administration of a cannabinoid antagonist (Colombo et al., 1998; Navarro et al., 2001) and the reward deficits observed in the CB1 receptor knockout mice (Ledent et al., 1999; Maldonado and Rodríguez de Fonseca, 2002; Sanchis-Segura et al., 2004). Research on the neurobiological basis of endocannabinoid effects on motivated behaviour has focused on endocannabinoid–dopamine interaction as well as on the role of the endocannabinoid system in habit learning and conditioning. The extended amygdala is the target of the ascending mesocorticolimbic projections of the ventral tegmental area (VTA) dopaminergic neurons, a subset of mesencephalic neurons that display a consistent response to drugs of major abuse, which appear to be a common substrate for the reward properties of drugs of dependence (Maldonado and Rodríguez de Fonseca, 2002). Most drugs of dependence activate the VTA dopaminergic neurons, as monitored by the dopamine release in terminal areas, especially in the nucleus accumbens and prefrontal cortex, or by the firing rates of VTA dopaminergic neurons. THC and other CB1 receptor agonists increase dopamine efflux in the nucleus accumbens and prefrontal cortex and increase the dopaminergic cell firing in the VTA (for review see Gardner and Vorel, 1998). This effect is not caused by the direct activation of dopaminergic neurons because they do not express CB1 receptors (Julian et al., 2003). Although the effects of cannabinoid agonists on dopamine release in the projecting areas (i.e. nucleus accumbens) can be blocked by the opioid antagonist naloxone, the increase in VTA dopaminergic cell firing cannot be blocked. This discrepancy may suggest the existence of a differential role for endogenous opioid systems as the modulators of cannabinoid actions in dopamine cell bodies with respect to their axon terminals. Cannabinoid effects might also involve glutamatergic and GABAergic inputs to the nucleus accumbens and VTA, because presynaptic CB1 receptors regulate glutamate and GABA release in these areas, inducing LTD (Schlicker and Kathmann, 2001; Robbe et al., 2002). In agreement with these actions of cannabinoids in brain reward circuits, repeated cannabinoid exposure can induce behavioural sensitization similar to that produced by other drugs of dependence. Chronic cannabinoid administration also produces cross-sensitization to the locomotor effects of psychostimulants (Maldonado and Rodríguez de Fonseca, 2002). Because endocannabinoids induce LTD in the nucleus accumbens (which affect glutamatergic inputs coming from the prefrontal cortex), they probably regulate the acquisition of habit learning and conditioned responses relevant to the progressive loss of control that characterize drug addiction (Maldonado and Rodríguez de Fonseca, 2002). Interestingly, administration of a CB1 receptor antagonist blocks cue-induced reinstatement to heroin and cocaine self-administration (De Vries et al., 2001, 2003). The importance of the endogenous cannabinoid system in the control of motivated behaviour goes far beyond the control of processing ongoing reward signals. The CB1 receptors are apparently involved in the control of reward homeostasis (Sanchis-Segura et al., 2004). Moreover, when cannabinoid homeostatic mechanisms are not adequate to restore the lost equilibrium in reward control derived from continuous uncontrolled exposure to a reinforcer (e.g. opiates or alcohol), allostatic changes involving CB1 receptors are set in motion to counteract the spiralling distress imposed on the reward circuit. This has been demonstrated in rodents exposed to cycles of dependence–abstinence to alcohol and morphine (Navarro et al., 2001; Rimondini et al., 2002). In this model, a history of dependence is associated with a permanent upregulation of the expression of CB1 receptors in reward-related areas and with an enhanced sensitivity to reward disruption induced by cannabinoid receptor antagonists (Rodríguez de Fonseca et al., 1999; Rimondini et al., 2002). Whether these allostatic changes occur in other models of motivated behaviour (i.e. feeding) remains to be determined.

Cannabinoid receptors are not only associated with motivational disturbances, but also related to emotional processing. A key station for the endocannabinoid regulation of emotions is the amygdalar complex. Endocannabinoids are able to depress the release of glutamate and corticotropin-releasing factor, reducing the amygdalar output and the activity of basolateral inhibitory GABA projections to the central nucleus of the amygdala, thereby activating the amygdalofugal pathway (Rodríguez de Fonseca et al., 1996, 1997; Navarro et al., 1997; Marsicano et al., 2002; Piomelli, 2003). The final balance will lead to anxiety or anxiolysis, depending on the rate of activation of descending projections of the central nucleus of the amygdala to the hypothalamus (endocrine responses) and brain stem (behavioural and autonomic responses). However, recent studies indicate that anxiolysis is the normal response to enhanced cannabinoid transmission in the limbic system, as reflected by the phenotype of FAAH knockout mice and the effects of FAAH inhibitors (Cravatt et al., 2003; Kathuria et al., 2003). The induction of anxiety by cannabinoid receptor antagonists (Navarro et al., 1997) supports this notion as well.

A practical approach: role for the endocannabinoid system in alcoholism

The presence of the endogenous cannabinoid system in reward circuits and its role in motivational and emotional homeostasis suggests that drugs which modulate cannabinoid signalling might serve as therapeutic tools in drug addiction. In accordance with this rationale, the CB1 receptor antagonists are able to modulate opioid self-administration in rodents (Navarro et al., 2001). Extending this hypothesis, converging research lines have established a role for both anandamide and the CB1 receptor in alcohol dependence (Hungund and Basaravajappa, 2000; Hungund et al., 2002; Mechoulam and Parker, 2003). The administration of CB1 receptor agonists promotes alcohol intake (Colombo et al., 2002), whereas the administration of a CB1 receptor antagonist decreases alcohol self-administration, especially in animals with a history of alcohol dependence (Rodríguez de Fonseca et al., 1999) or in alcohol-preferring rat lines (Colombo et al., 1998). Molecular studies have shown that chronic alcohol administration is associated with an increased formation of both anandamide and its membrane precursor NAPE (Basavarajappa and Hungund, 1999). Chronic alcohol exposure also resulted in the stimulation of a second endocannabinoid, 2-AG (Basavarajappa et al., 2000). Animal studies also revealed that chronic exposure to alcohol downregulated the CB1 receptors in the brain (Basavarajappa et al., 1998). Finally, a recent gene screening study has identified the CB1 receptor as one of the genes whose expression is permanently affected by serial cycles of alcohol dependence and withdrawal (Rimondini et al., 2002). These data indicate a role for the endogenous cannabinoid system as a relevant contributor to alcoholism. Human gene studies support this experimental hypothesis, since a linkage between clinical forms of alcoholism and polymorphisms and/or mutations of the genes encoding either the CB1 receptor (Comings et al., 1997; Schmidt et al., 2002) or the FAAH (Sipe et al., 2002), the enzyme responsible for AEA inactivation (Cravatt et al., 1996), have been described. In the present issue, the reader will find additional experimental approaches to the role of the endogenous cannabinoid system in alcoholism.

CONCLUSION

Since the discovery of anandamide, the increasing information on the physiological roles played by the endogenous cannabinoid system and its contribution to pathology have led to this signalling system becoming more important in neurobiology. The intense pharmacological research based on this information has yielded, in a very short time, potent, selective drugs targeting the endogenous cannabinoid system that have opened up new avenues for the understanding and treatment of major diseases including cancer, pain, neurodegeneration, anxiety and addiction. This is a very promising starting point for a new age that takes over from the ancient use of Cannabis as a medicine. Now is the time for clinical trials aimed at evaluating the efficacy of cannabinoid drugs in disorders lacking effective therapeutic approaches, such as alcoholism.

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View Abstract

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Cannabinoids
58
The therapeutic components of the cannabis plant are contained in the resinous glands (called trichomes) that exist on the plant surface, with the highest density on the female flower. One of the medically active chemical categories in the resinous glands is a group of plant chemicals called cannabinoids or phytocannabinoids. There are over 70 cannabinoids found in the cannabis plant. The most well known cannabinoids is the primary psychoactive chemical in cannabis, delta-9-tetrahydrocannabinol (THC). While THC is what makes cannabis popular as a recreational drug, it is but one of over 400 compounds derived from the plant that may contribute to its therapeutic effect (Guzman, 2009).
Endocannabinoids
"Research on its psychological effects [that of the cannabis plant] led directly to the discovery of a new chemical signaling system in the human body which is now recognized as playing a crucial role in regulating our neurology and physiology (Werner, 2011, p. 9)." Would it surprise you to know that cannabinoids exist in the human body? They also exist in most living creatures, and possibly even in single-celled-organisms. Endocannabinoids are produced in the human body to help govern the physiological process of:

This picture show the ultra magnification of the surface of the cannabis flower. The milky white straws shooting away from the surface of the plant are called trichomes. This is where the pharmocological properties of cannabis reside.
• pain modulation
• immune function
• newborn suckling
• appetite reward
• thermoregulation
• memory
• inflammation regulation (Abrams, 2011).
This new found understanding of cannabis, and the biological systems of the human body, and their integration provides profound opportunity for advances in managing and altering the disease process for a wide variety of illnesses. Clint Werner nails it in his book Marijuana Gateway to Health, when he writes, "Marijuana is beginning to emerge from under a cloud of contempt because it has been at the center of one of the most exciting and under appreciated developments in biological science in recent times."
In 1988 the first cannabinoids receptor (molecule on the outside of a cell that receives a signal for the cell to do something specific) known as CB1, was discovered in humans. The receptors reside primarily in the brain with the highest concentration in the basal ganglia, the hippocampus, and cerebellum (Guzman, 2009). This receptor is the one that receives THC thereby activating its euphoric properties. Additionally, the function of the CB1 receptor is for appetite, immune function, muscle control, pain, inter-ocular pressure, cognition, reward mechanism, thermoregulation, and emesis (nausea and vomiting) (Abrams, 2011).
In 1993, a second cannabinoid receptor was found in the macrophage (type of white blood cell) cells in the spleen with the highest concentrations in the natural killer cells strongly suggesting a role in immune function, cell proliferation, inflammation and pain (Guzman, 2009).
Cannabis Science
Recent science shows that different cannabinoids and other components in cannabis, which have little to no psychotropic effect, may actually hold more therapeutic promise than THC. (Angelo A. Izzo Francesca Borrelli Raffaele Capasso, 2009). A few of the most studied cannabinoids beyond THC are Cannabidiol (CBD), cannbichromene (CBC), cannabigerol (CBG), tetrahydracannabvarin (THCV), cannbidivarin (CBDV), cannabinol (CBD). Each of these phytocannabinoids have multiple benefits to improving the health of the human body. See Dustin Sulak's eloquent Introduction to the Endocannabinoid System on the NORML website for a great overview of the endocannabinoids system.
Tetrahydracanabinol (THC)
While THC, the most common cannabinoid in cannabis, is known for the high people get from inhaling/ingesting marijuana, it also relieves pain and inflammation and is an anti-spasmodic and muscle relaxant. In clinical trials, THC has been proven to reduce levels of vascular endothelial growth factor (VEGF) in glioma (brain cancer) cells. This is important because a reduction in VEGF means that brain cancer tumors are unable to grow the new blood vessels they need to sustain themselves, grow and spread. This discovery offers great promise for the treatment of brain cancer (Blázquez, González-Feria, Alvarez, A, Casanova, & Guzmán, 2004).
THC is also a powerful neuroprotective, antioxidant and has 20 times the anti-inflammatory power of aspirin and twice that of hydrocortisone (Russo, 2011).
Cannabidiol (CBD)
CBD is the second most common cannabinoid in medical marijuana and the most common in hemp plants (Russo, 2011). CBD rich cannabis provides potent therapeutic benefits without the euphoria or lethargy of many high THC varieties. CBD and THC are believed to have synergistic effect meaning that when they are both present at therapeutic levels they are more effective together than they are alone. While THC is widely known for its psychoactive properties, CBD may be best known for its ability to counterbalance anxiety, tachycardia, hunger and sedation caused by THC (Russo, 2011).
CBD, along with other cannabinoids was once considered a minor cannabinoids under the shadow of THC. What we know now is that CBD is as beneficial and versatile a cannabinoid as THC in addressing many hard to manage conditions such as diabetes, rheumatoid arthritis, cancer, epilepsy, antibiotic-resistant infections, alcoholism, PSTD and neurological disorders (O'Shaugnessy's News Service, 2011). CBD is different however in that it does not have a strong binding affinity for the CB1 and CB2 receptor, thus it works in the body through different direct and indirect pathways. For a more thorough explanation of how and why CBD works in the body, read the article CBD: How it Works by Martin A. Lee. The following is a basic list of how CBD can benefit the human body:

• Potent anti-oxidant that is stronger than vitamins C and E. The combination of THC and CBD make an even stronger anti-oxidant (Lee, 2011).
• Powerful analgesic that seems to work especially well for traditionally difficult to treat neuropathic pain (Lee, 2011).
• Neuroprotective agent that is thought to help prevent Alzhiemer's and other age related neurodegenerative disorders (Lee, 2011).
• Serotonin balancing agent which acts as an antidepressant, and has a positive impact on reducing anxiety, addiction, appetite, sleep, pain perception, nausea and vomiting (Lee, 2011). Serotonin also affects your ability to rest, regenerate and find serenity (Eric Braverman, 2004, p. 138)
• Regulates blood pressure.
• Facilitates bone re-absorptions.
• Cytotoxic to breast cancer and other cancer cells while being a cyto-preservative to healthy cells. (Russo, 2011)
• Agent against methicillin-resistant Strephylococcus aureus (MRSA), the staph infection that plagues many hospitals today (Lee, 2011).

While there is so much more to learn about CBD and how and why it works in the body, there is enough compelling evidence that leads researchers to believe that whole plant cannabis therapeutics offers great promise for society. One day, CBD will likely become a powerful and widely accepted healing agent used for many different ailments.

Unfortunately, CBD has largely been bred out of much of the cannabis available in the U.S., U.K. and Europe while THC content has been increased because of past emphasis on recreational use. Thanks in part to a group called Project CBD and its supporters, we now know that about one in every 750 samples being tested in California are CBD rich strains (Project CBD, 2012). CBD rich strains are those with 4 percent or higher CBD content. This information makes it possible for the pendulum to swing the other way towards more therapeutic balance or at the very least makes it easier to breed and grow plants that are rich in CBD. As more data becomes available, cannabis breeders/growers around the world are striving to grow marijuana with higher CBD levels to create better medicine. To learn more about Project CBD and support their research efforts please visit their website at www.projectcbd.org.

Cannabinol

Cannabinol (CBN) is a lesser understood cannabinoid. Scientific research is frequently done first on rats, monkeys and other animals before doing so in human models. To date, there is conflicting research on CBN between the animal and human models. However, what we do know is that CBN is usually found in aging cannabis and increases the effect of THC. CBN increases testosterone production and plasma concentration of follicle-stimulating hormone. It is also an anti-convulsant and anti-inflammatory. Because CBN has a three-times greater affinity for the CB2 receptor, it s believed to have a greater effect on the immune system than the central nervous system (John M. McPartland, 2001).

Cannabichromeme

Cannabichromene (CBC) while it is the fourth major cannabinoid, it is generally found in only tropical strains of cannabis. It is thought to be superior to THC and CBD in its antifungal and antibacterial activity showing great promise for topical and internal uses. On the flip side, while CBC does inhibit the cellular inflammatory response like THC and CBD, it does not do so at the same effectiveness (John M. McPartland, 2001, p. 108).

Cannabigerol

Cannabigerol (CBG) is a potent antibacterial that is proven superior to THC, CBD and CBC against gram-positive bacteria, mycobateria and fungi. Although CBG exists in very small amounts in cannabis, it is the biosynthetic precursor to THC, CBD and CBC meaning the body will convert CBG to another cannabinoid.

Tetrahydrocannabivarin

Tetrahydrocannabivarin (THCV) is found in indica and afagnica varieties of cannabis. While this is not a primary cannabinoid it is important for two primary reasons: it is being considered at a biochemical marker for illicit drug use because it is not a metabolite of Marinol and easily detectable in drug testing and it may be clinically effective in the treatment of migraine headaches (John M. McPartland, Cannabis and Cannabis Extracts: Greater Than the Sum of Their Parts?, 2001)

Taming T

HC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects

From <http://mychronicrelief.com/cannabis-science/cannabinoids/>

Modulation Of The Endo-Cannabinoid System: Therapeutic Potential Against Cocaine Dependence

Gianluigi Tanda

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Abstract

Dependence on cocaine is still a main unresolved medical and social concern, and in spite of research efforts, no pharmacological therapy against cocaine dependence is yet available. Recent studies have shown that the endocannabinoid system participates in specific stages and aspects of drug dependence in general, and some of this evidence suggests an involvement of the cannabinoid system in cocaine effects. For example, cocaine administration has been shown to alter brain endocannabinoid levels, and the endocannabinoid system has been involved in long-term modifications of brain processes that might play a role in neuro/behavioral effects of psychostimulant drugs like cocaine. Human studies show that marijuana dependence is frequently associated with cocaine dependence, and that the cannabinoid receptor CNR1 gene polymorphism might be related to cocaine addiction. This article will review the main papers in the field showing how a modulation of different components of the cannabinoid system might interact with some of the neurobiological/behavioral effects of cocaine related to its reinforcing effects, evaluated in preclinical models or in clinical settings. The goal of this review will be to provide insights into the complex picture of cocaine abuse and addiction, and to extrapolate from such endocannabinoid-cocaine interactions useful information to test the therapeutic potential of cannabinoid ligands and endocannabinoid-level enhancers against cocaine dependence for future preclinical/clinical trials.
Keywords: cannabinoids, cocaine, addiction, behavior
Go to:

1. INTRODUCTION

A number of studies suggest that the cannabinoid system interacts with cocaine's effects at different levels in the brain. For example, both CB1 agonists and cocaine selectively increase dopamine neurotransmission in the nucleus accumbens shell (1, 2, 3, 4), a brain area believed to play a pivotal role in the addictive effects of drugs (5). It is also interesting to note that cocaine administration results in increased levels of anandamide, one of the endogenous ligands for cannabinoid receptors in the brain (6, 7), and that blockade of CB1 cannabinoid receptors attenuates a dopamine signal triggered by cocaine in the NAc (8).

The cannabinoid system has also been implicated in brain processes related to potentiation or depression of the strength of synaptic signals (9, 10, 11, 12). These so-called processes of long term potentiation and long-term depression activated by cannabinoid neurotransmission might play a significant role in those neurobiological adaptations which are believed to take an important part in the road leading from drug use to drug abuse and addiction (13, 14, 15). Moreover, cannabinoid receptors are located in brain areas that have been assigned a key role in brain functions linked to the development of drug-dependence (16). Plant-derived and endogenous cannabinoid ligands have been suggested to affect neurotransmitter transporter functions (17, 18, 19, 20, 21, 22), which are also pharmacological targets for cocaine (23, 24). Finally, it is interesting to note that cocaine dependence is often diagnosed in the presence of other drug dependencies (25), and marijuana dependence is second only to alcohol dependence as the most frequent comorbid dependence in cocaine addicts (25, 26).

1.1 Overview on cocaine abuse and addiction

Cocaine abuse and addiction are widespread throughout the world, and these phenomena are driven by the ability of this drug to produce reinforcing effects by interacting with specific sites in the central nervous system (23). Cocaine binds to specific transport sites on the cell membranes of neurons that release dopamine, serotonin or noradrenaline. These transport sites have the physiological function of removing neurotransmitters from the extracellular space after their release from nerve terminals (24). Binding of cocaine to the neurotransmitters' transport sites results in blockade of the transport, increased availability of neurotransmitters for binding to their respective receptors, and ultimately increased neurotransmission (23, 27). Although cocaine also binds and blocks the transporters for serotonin and noradrenaline (24), blockade of the dopamine transporter (DAT) appears to be the pharmacological target of cocaine mainly related to its reinforcing and other behavioral effects (23, 27). Thus, increased availability of dopamine for dopamine receptors is believed to mediate most of the reinforcing actions of cocaine, leading to its abuse liability (1, 3, 23, 27). However, cocaine abuse and addiction are processes that go well beyond the pharmacological effects of cocaine on dopamine neurotransmission, so that many other factors, genetic and environmental for example, have been suggested to play a role in the complex of actions that lead from cocaine use to its abuse and addiction (28, 29, 30, 31).

There is no specific pharmacological therapy available against cocaine's addictive effects, but researchers in the last decades have made progresses in the understanding of the neurobiology underlying cocaine's actions (29, 30). Today, different aspects of cocaine abuse and addiction in humans can be modeled and studied in experimental animals. These animal models have provided valuable results leading to a better understanding of the different factors contributing to cocaine effects (32). Moreover, the models have been invaluable in the study of newly synthesized compounds acting over different neuronal systems that might become candidates for development as cocaine abuse medication (29, 30).

1.2 Overview on the brain endocannabinoid system

The important discoveries in the last two decades in the cannabinoid field have provided useful information about the physiological functions of the endocannabinoid system in the brain and periphery, and the involvement of this system in the etiology and therapy of many human diseases (33, 34, 35). During the last decade researchers have provided evidence for the existence of at least two cannabinoid receptors, CB1 and CB2 (36, 37, 38, 39) and we have now available different selective synthetic agonists and antagonists for both CB1 and CB2 receptors (40, 41), other than classic, plant-derived cannabinoid agonists like delta-9-THC, the active ingredient in marijuana. Although the existence of novel cannabinoid receptors has been suggested in different studies (42, 43, 44), definitive proof of the existence of these cannabinoid receptors has yet to be shown.

Several different endogenous ligands for CB1 and CB2 receptors have been found, such as anandamide, 2AG, noladin-ether (45, 46, 47, 48). At variance with classical neurotransmitters, these endocannabinoids are not stored in vesicles in nerve endings, but are released "on demand" by specific changes occurring in neurons which promote the function of specific enzymes that produce anandamide or 2-AG from membrane-constituent lipids (49, 50, 51, 52). We now have evidence that endogenous cannabinoids released "on demand" can function as retrograde messengers in the synapse (53). Thus, activation of cannabinoid receptors might change the electric equilibrium of the post-synaptic membranes with important modifications for cell-depolarization, neuronal firing, and release of classic neurotransmitters, which ultimately results in a modification of brain neurotransmission (10, 11).

Some of the neurobiological effects of endocannabinoids have been demonstrated to be long-lasting, leading to the so-called processes of long-term potentiation and long-term depression of the synaptic transmission strength (11, 12). It is interesting to note that these long-term effects are in spite of the very brief half life of endogenous cannabinoid ligands in the extracellular space (49, 54, 55).

Anandamide and 2-AG are taken-up into the cell by a not completely defined and still debated mechanism/s: passive diffusion or facilitated transport across the plasma membrane (55, 56, 57, 58, 59). Anandamide is then metabolized into the cell by an intracellular enzyme, the fatty acid amide hydrolase (FAAH) (60, 61), the blockade of which has been shown to prolong the behavioral/neurobiological effects of anandamide (4, 61, 62, 63). In contrast, 2-AG is metabolized through the mono glyceril lipase enzyme (54). In the last few years several pharmacological tools have been discovered that alter the physiological equilibrium of the endocannabinoid system by acting on the metabolic pathways of the endogenous ligands. The result of this modulation is that blockade of endocannabinoid metabolism increases levels of endocannabinoids in specific brain areas in which there is a preexisting cannabinoid tone or where endogenous cannabinoids are released on demand triggered by specific neuronal signals (34, 61). These highly-specific effects contrast with the effects of exogenously administered cannabinoid agonists, which diffuse widely throughout the brain leading to a generalized and contemporaneous activation of multiple brain areas.

It is believed that in humans, cannabinoid receptor agonists, like delta-9-THC, activate cannabinoid CB1 receptors, producing reinforcing effects which are the basis for marijuana abuse (2, 64). CB1 receptors are also involved in other brain mechanisms and functions associated with pathologies in which a cannabinoid intervention might have a therapeutic value (33, 34, 65, 66). Unfortunately, there is no cannabinoid agonist drug available yet that can selectively produce therapeutic effects without having undesired psychotropic, reinforcement-related effects. However, pharmacological tools are now available that can modulate the activity of endogenous cannabinoid ligands in selected brain areas, thereby having therapeutic value (34) without the harmful reinforcing effects produced by delta-9-THC-like drugs with the widespread action. Indeed, interesting actions of such cannabinoid modulators have been already described in preclinical models of anxiety (61, 67) and depression (68), and preclinical tests so far have also shown negative results in procedures involving models of drug abuse (4, 68, 69).

Go to:

2. Effects of cannabinoid drugs or endocannabinoid modulators in preclinical models of cocaine's behavioral and reinforcing effects

2.1 Cannabinoid interactions with cocaine-induced stimulation of motor activity and behavioral sensitization

2.1.1 Effects after acute administration of cocaine

Psychostimulants, like cocaine, have the ability to influence neuronal activity, which results in stimulation of behavioral activity. Stimulation of locomotor activity induced by cocaine and other psychostimulants has been extensively studied (see, for example citations 70, and 71). Administration of psychostimulants in rodents induces a dose-dependent increase in behavioral activation, which depends on the activation of selected brain striatal areas (72, 73, 74, 75, 76), and that is accompanied by stimulation of DA neurotransmission (3, 77, 78, 79). Although this behavior is not a direct correlate of the reinforcing effects of cocaine, the fact that it can be obtained by injecting cocaine in the NAc (72, 76, 77), and that NAc DA transmission is involved in different aspects of drug abuse and addiction (5, 80, 81), makes monitoring of locomotor activation an easy preclinical target to test drugs that can interfere with the ability of cocaine to stimulate DA neurotransmission and potentially attenuate cocaine-induced reinforcement (82).

Do cannabinoids alter the acute effects of cocaine on stimulation of motor activities?

Pryor and colleagues, 1978 (83), described the interaction between cocaine and delta-9-THC on motor activity, measured as photo-beam interruptions in rats, as one of mutual antagonism dependent on the combination of doses tested. The antagonism was attenuated but still evident after sub-chronic administration of delta-9-THC. In agreement with these data, Ferrari et al., 1999 (84), showed that HU210, a synthetic agonist for CB1 receptors, dose dependently reduced the locomotor activity induced by cocaine, 15 mg/kg i.p. in rats. However, sub-chronic administration of HU210 was not able to modify cocaine-induced locomotion at variance with the previous report. It should be noted that a cannabinoid antagonist was not available in 1978, and that Ferrari et al., 1999 (84), did not test whether a CB1 receptor antagonist could interfere with HU210 actions on cocaine effects. Because of the lack of direct pharmacological involvement of CB1 receptors in cocaine effects, Przegalinski et al., 2005 (85), tried to address this issue in a more recent report. In that study the authors showed that the CB1 receptor agonist WIN 55,212-2, but not its less active enantiomer WIN 55,212-3, dose dependently (3–6 mg/kg i.p.) reduced cocaine-induced hyper-locomotion, without affecting behavior when administered alone. Rimonabant (SR 141716), a selective antagonist of CB1 receptors (86), did not reverse the effects of WIN 55,212-2 on cocaine-stimulated locomotion, suggesting a non-CB1 mediated mechanism of action for WIN 55,212-2. Moreover, the effect was not attributable to unspecific interactions of the highly lipophilic cannabinoid agonist WIN 55,212-2 with hydrophobic sites of proteins or other cell's membrane lipid constituents, since the less active enantiomer did not show any significant effect on cocaine-induced hyper-locomotion (85). Further, the authors showed that the effects of WIN 55,212-2 resembled those of a serotonin 5HT3 antagonist, based on other previously published evidence (87, 88), thus indicating the possibility of an involvement of 5HT3 receptors in the action of cannabinoid agonists on cocaine-induced hyper-locomotion. However, other than a resemblance of effects, there was no direct pharmacological evidence for 5HT3-receptor involvement in the actions of WIN 55,212-2 on cocaine-induce hyper-locomotion (85). It should also be noted that in the report by Przegalinski et al., 2005 (85), the locomotor stimulant effects of cocaine were reduced to a certain extent, though not significantly, by Rimonabant itself, while in a recent report (8) Rimonabant significantly attenuated cocaine-induced hyperactivity, thus suggesting the possibility that both agonist and antagonists of CB1 receptors can negatively modulate the locomotor stimulant effects of cocaine. Also, Rimonabant antagonized cocaine-induced hyper-locomotion in Gerbils habituated to the test cage, but not in non-habituated animals (89). The authors explained their results in terms of a cannabinoid involvement as a permissive element required in the ability of cocaine to override novelty-stimulus satiation in habituated Gerbils. However, lack of effects of Rimonabant on acute cocaine-induced locomotor stimulation has also been reported in habituated mice and rats (90, 91).

Taken together these experiments suggest that an endocannabinoid tone might not be necessary to mediate the acute motor stimulant effects of cocaine in rodents. However, depending on dose, both cannabinoid CB1 receptor agonists and antagonists might modulate this behavioral stimulation induced by cocaine. Moreover, as shown by Cheer et al., 2007 (8), attenuation of cocaine-induced locomotor activity by a CB1 receptor antagonist has a neurochemical correlate. In that study, Rimonabant pretreatment attenuated dopamine neurotransmission, as measured by "fast-scan-cyclic voltammetry", suggesting that a cannabinoid tone might be involved in both neurochemical and behavioral processes triggered by cocaine (8).

2.1.2. Effects in cocaine-sensitized animals

Psychostimulant sensitization is a phenomenon that has been extensively described in rodents. It appears as an increased effectiveness of the psychostimulant drugs to produce behavioral activation in animals previously exposed to single (92) or repeated (93) administration of the same drug. Cross sensitization between different drugs has also been described (94, 95).

It has been suggested that sensitization induced by drugs of abuse may be due to neuronal adaptations that play a role in the development of addictive behaviors. Behavioral sensitization is easily demonstrated in animals, and it has been hypothesized that this adaptation is involved in the transition from drug use to abuse and addiction (see citations 14, and 15, for review). Thus, behavioral sensitization has been the target of different studies searching for drugs able to impair or reduce the effects of this adaptation induced by sub-chronic/chronic administration of cocaine.

Do cannabinoids play a role in the behavioral sensitization induced by cocaine?

There are only a few reports about cannabinoid interactions with development or expression of cocaine-induced behavioral sensitization. Sensitization and cross sensitization have both been reported for cannabinoid agonists and some drugs abused by humans including psychostimulant drugs (94, 95, 96, 97, 98, 99, 100). However, failure to develop cross sensitization in rats chronically treated with a cannabinoid agonist, CP 55,940, and challenged with cocaine has also been reported (101). In the same study, CP 55,940 failed to enhance the sensitivity to cocaine when the drugs were concurrently administered during the development phase. Also, the development of cocaine sensitization did not appear to be under control of CB1 receptors in rodents in another report (90). In this latter study, mice were treated with 20 mg/kg cocaine for 11 days in their home cages. Three days after the last cocaine injection they habituated to an open field, and then tested with cocaine (10 mg/kg). The authors reported a first set of experiments in which Rimonabant 1 mg/kg administered 30 min before the daily cocaine injection significantly reduced the locomotor sensitization induced by cocaine. Such effects, however, were not replicated in a subsequent set of experiments with increasing doses of cocaine (90).

In genetically modified CB1 receptor knock out mice (102), cocaine (10 mg/kg twice daily for 15 days) induced a behavioral sensitization, tested after one week withdrawal in an open field, which was indistinguishable from that induced in wild-type littermate control mice. These findings once more suggest a lack of involvement of the endocannabinoid system in the development of behavioral sensitization induced by cocaine. On the other hand, the expression of cocaine sensitization has been demonstrated to be impaired by cannabinoid CB1 receptor blockade, as shown by administration of the selective CB1 receptor antagonist Rimonabant together with cocaine five days after withdrawal from cocaine (103). Although this interaction needs to be further investigated, results from the cited reports suggest that even if activation of cannabinoid neurotransmission through CB1 receptors might not be required for development of cocaine-induced sensitization, an endogenous cannabinoid tone might be essential for its expression.

Thus, cannabinoid antagonists might decrease the impact of a "sensitized" response to drug stimuli in subjects during abstinence. This prediction matches the results obtained by DeVries et al., 2001 (104), with administration of the cannabinoid receptor antagonist Rimonabant in cocaine abstinent rats during self-administration reinstatement tests (see below the section: "Effects of cannabinoids on animal models of extinction and reinstatement of cocaine-seeking behavior").

2.2 Cannabinoid interactions with cocaine-induced place conditioning

The place conditioning procedure is a model of drug reinforcement and is based on an individual's behavioral choice after a certain number of pairings of drug with one environment and placebo with another. This procedure, though simple, can give an indication of the pleasurable (place preference) or aversive (place avoidance) feelings/perceptions induced by drugs during the conditioning procedure. The place-conditioning allows the study of both development and expression of aversion or preference for a place associated with a certain drug dose (see citations 105, and 106 for review), with the final test performed with animals in a drug-free state. So, the ability of drugs to interfere or impair the effects of the conditioning-drug could be related to a potential therapeutic value, in terms of ability to reduce or block the pleasurable feelings induced by the conditioning drug, or to attenuate the strength of the association between the conditioning drug and the environmental stimuli.

It has been repeatedly shown that cocaine, just like many other drugs abused by humans, supports place preference at certain doses (106). On the

other hand, results from place conditioning studies with cannabinoid agonists and antagonists have been inconsistent (see citation 107 for review), and both preference and aversion induced by cannabinoid CB1 receptor agonists and antagonists have been reported under a variety of different experimental conditions and dose ranges (108, 109, 110, 111, 112, 113).

The involvement of the cannabinoid system in processes related to positive reinforcement induced by cocaine in the place conditioning procedure was evaluated by Chaperon et al., 1998 (91), in rats. The authors showed clear evidence that blockade of CB1 receptors by administration of the selective cannabinoid CB1 receptor antagonist Rimonabant impaired the acquisition of cocaine-conditioned place preference, suggesting that the development of such conditioning was under control of a cannabinoid tone in the brain. In the same paper the authors also showed that blockade of CB1 receptors did not impair the expression of cocaine-conditioned place preference when the antagonist of CB1 receptors, Rimonabant, was administered only once, 30 min before the final test, instead of 30 min before each cocaine injection during the conditioning sessions (91). At variance with this report, results from a study by Martin et al., 2000 (102), showed that development and expression of cocaine-conditioned place preference can be established in genetically modified CB1 receptor knock-out mice. These latter results suggest no involvement of the endogenous cannabinoid systems in brain processes related to the motivational effects induced by cocaine in this procedure in genetically modified mice (102). These contradictory findings (91, 102) might be explained on the basis of species differences, or developmental adaptations in the regulatory processes involved in the mediation of cocaine reinforcing effects in mice genetically deficient in CB1 cannabinoid receptors. It should be noted that Rimonabant might have inverse agonist actions on CB1 receptors (114, 115, 116), or might possess actions over pharmacological targets other than on CB1 receptors, for example on the putative CB3 receptor (42, 65). Martin and colleagues (102) did not cite the article by Chaperon et al., 1998 (91), so we do not have their comments on this issue. We also do not have information about the effects of Rimonabant administration on development and expression of cocaine-induced place preference in wild-type mice, an experiment that would allow a direct comparison with data obtained in CB1 KO mice (102).

2.3 Effects of cannabinoids on the discriminative stimulus effects of cocaine

The drug discrimination procedure has been extensively used in animal and human subjects to evaluate the specific perceptions/feelings produced by drug administration (117). It is likely that these subjective effects play an important role in the reinforcing effects of many drugs abused by humans. Indeed, many of these drugs produce euphoric or pleasurable sensations that are believed to be factors contributing to the abuse of substances (117). The discriminative stimulus properties of cocaine have been evaluated in many differents studies under different conditions and in different species, including human subjects (117, 118). However, there is a very limited set of data available in which the influence of the cannabinoid system has been evaluated in animals trained to discriminate the subjective effects of cocaine from those of placebo. Delta-9-THC did not substitute for cocaine in pigeons trained to discriminate cocaine from placebo, or cocaine from morphine, suggesting that the discriminative stimulus of the cannabinoid agonist and that of cocaine do not overlap (119, 120). In a more recent report (103) the cannabinoid receptor antagonist Rimonabant did not substitute for cocaine, and did not change significantly the discriminative stimulus effects of cocaine in rats.

These limited findings indicate that the endogenous cannabinoid tone mediated by CB1 receptors does not play a role in the expression of the discriminative stimulus of cocaine. However, more detailed studies with a complete range of cannabinoid drugs (agonist, antagonist, and modulators) and doses are needed to further elucidate the role of the cannabinoid system in the subjective effects of cocaine.

2.4 Cannabinoid modulation of the effects of cocaine on thresholds for Intra Cranial Self Stimulation (ICSS)

This procedure is based on studies by Olds and Milner (121), in which rats have the opportunity to learn to repeatedly press a lever in order to obtain an electrical stimulation of the medial forebrain bundle, a component of reward circuits in the brain (122). It has been repeatedly shown that most drugs of abuse are able to lower ICSS thresholds in a dose-dependent fashion (122, 123). This effect has been used as an indirect measure of their ability to produce “reward” through brain processes linked to the medial forebrain bundle. Thus, substances able to reduce or block the ability of drugs of abuse to lower the ICSS threshold may have therapeutic potential as treatments for drug abuse. While the effects of cocaine in this procedure are consistent with its reinforcing effects in animal and human subjects, inconsistent results have been obtained with administration of cannabinoid drugs (124, 125; see also citations 106, and 126, for review). There is only limited information available for combinations of cocaine and cannabinoids studied with the intracranial self stimulation procedure. In a study by Pradhan et al., 1978 (127), co-administration of delta-9-THC in rats resulted in an antagonism of the effects of cocaine, while in experiments in which delta-9-THC was administered 80 min before cocaine, the effects of cocaine were unaffected by the cannabinoid agonist. In agreement with this study, in a more recent report (128) administration of the synthetic cannabinoid agonist WIN 55,212-2 reduced the effects of cocaine on ICSS thresholds. In the same study, administration of the CB1 receptor antagonist, Rimonabant, antagonized the effects of WIN 55-212,2 on cocaine. Also, Rimonabant administration did not affect cocaine actions on this procedure, though only very low doses of the CB1 antagonist were tested (128).

These above cited reports suggest that cannabinoid agonists might have some potential activity against cocaine reinforcing effects in rodents, however, the mechanism/s for these interactions are not clear. Drugs of abuse and intra cranial self stimulation produce reinforcing effects through the same reward circuitry (122), and also increase dopamine neurotransmission in a dopaminergic reward-related area of such circuitry (1, 2, 3, 78, 129, 130) that has been related to their reinforcing effects (5, 79, 80). Thus, it is not apparently clear why cocaine and cannabinoid CB1 receptor agonists, which share the ability to stimulate the brain reward circuitry, may have different and antagonistic effects in this procedure. However, the fact that in rodents cannabinoid agonists do not consistently produce reinforcing effects, but do so only under certain experimental conditions (108, 124, 131, 132, 133, 134; see also 107, 126, and 135), suggests a cautious approach to the outcome of experiments involving reinforcing effects of cannabinoids in these species as compared to the well known consistent reinforcing effects of marijuana in human subjects.

2.5 Effects of cannabinoids on cocaine-maintained self-administration behavior

Although the most direct outcome of this procedure is to predict reinforcing effects and abuse liability in humans for drugs that maintain self-administration behavior in experimental animals, many other aspects of this behavioral procedure can contribute to a better understanding of the behavioral effects of drugs and drug-related cues, their related neurobiology and potential therapeutic interventions (136, 137).

As a drug that is widely abused, cocaine consistently maintains intravenous self-administration behavior across a range of doses in a variety of different species (see, for example, citations 138, and 139). Although the reinforcing effects obtained by administration of plant derived cannabinoids in humans were described in books thousands of years ago, cannabinoid agonists do not consistently maintain self-administration behavior in experimental animals (see citations 107, 126, and 135 for review).

Preclinical studies have shown that pharmacological alterations of the endocannabinoid receptor system might interfere with the ability of cocaine to maintain self-administration behavior. In Long Evans rats, administration of the cannabinoid agonist WIN 55-212,2 reduced the average number of intravenous self-injections of different doses of cocaine under a fixed-ratio 1 schedule, suggesting a potentiation of cocaine effects (140). In the same report, the selective antagonist of CB1 receptors Rimonabant antagonized the effects of WIN 55-212,2 on cocaine self-administration behavior. Rimonabant, however, did not affect the behavior maintained by cocaine when administered alone (140). In agreement with this report, Rimonabant pretreatment did not show any effect on cocaine self-administration under fixed-ratio schedules of behavior in squirrel monkeys (63), an effect that has been recently replicated and confirmed (141, 142). In addition, Rimonabant did not affect the number of cocaine self-injections in Wistar rats studied under a fixed-ratio 5 schedule of drug delivery (103, 104). In agreement with the previous experiments in rats and primates, pre-treating C57Bl/6J mice with Rimonabant before single one-day sessions of cocaine self-administration affected neither the number of nose-pokes maintained nor the total cocaine intake during the 30-min session (90). In contrast the effects of genetic deletion of CB1 receptors have not been entirely consistent, and can differ with the effects of administering the pharmacological antagonist of CB1 receptors, Rimonabant. For example, using the single one-day cocaine self-administration procedure mutant cannabinoid CB1 receptor knock-out mice failed to self-administer morphine, but were still able to self-administer cocaine, suggesting that the endocannabinoid system was a substrate for mediating the reinforcing effects of opioids but not those of cocaine (143). At variance with these findings, a more recent report, using the same mutant cannabinoid CB1 receptor knock-out mice as subjects, showed a reduced number of mice reaching the acquisition criteria, which was also obtained within a lager number of sessions, and a significantly reduced reinforcing efficacy of cocaine (measured as the effort required to obtain a cocaine infusion) as compared to wild-type littermate control mice (144). The authors explain their results, contrasting with those of Cossu et al., 2001 (143), in terms of different experimental conditions; freely moving mice chronically receiving cocaine self-infusions in daily sessions (144) versus mice with restrained mobility receiving cocaine during

a single session (143).
Soria and colleagues (144) confirmed their data in mutant CB1 receptor knock-out mice studying the effects of pretreatments with the antagonist of CB1 receptors Rimonabant in wild-type mice on cocaine self-administration under a progressive-ratio schedule. They found that Rimonabant dose-dependently reduced the breaking point achieved by wild-type mice under the progressive-ratio schedule of cocaine self-administration, suggesting that also in normal control mice the endocannabinoid system was a substrate for the reinforcing effects of cocaine (144). It is interesting to note that in this report the authors excluded any involvement of learning/memory processes in the effects of cocaine in mice lacking the CB1 receptors because the same mice successfully acquired an operant response maintained by natural reinforcers (food and water). Also, the reduced efficacy of cocaine as a reinforcer was not the result of a diminished ability of cocaine to stimulate dopamine neurotransmission, measured by intra-cerebral microdialysis in limbic areas of mutant mice as compared to their littermate controls (144). These latter results point toward a more complicated and central involvement of the endocannabinoid system in the addictive effects of cocaine.
As neither deletion nor blockade of CB1 receptors impairs cocaine's ability to stimulate DA neurotransmission, it seems that the effects of modulation of the endocannabinoid system on cocaine self administration may be indirect. In a recent report using Sprague Dawley rats, prior exposure to delta-9-THC did not affect the acquisition and subsequent rates of cocaine self-administration under a fixed-ratio 1 schedule of injection, but did reduce the number of "cocaine-seeking" responses under a progressive-ratio schedule (145). Thus the sensitivity to delta-9-THC exposure may vary with behavioral procedure. Indeed, it has been already reported that changes in reinforcing efficacy of cocaine are hard to detect using fixed-ratio schedules of cocaine self-administration (146). In the report by Panlilio and colleagues (145), delta-9-THC pre-exposure also decreased both the number of entries in the center-zone of an open-field arena, and the time spent in the lighted compartment of the dark-light box (two different predictors of an anxiety response) in cocaine-treated rats. The authors suggested that the reduced effectiveness of cocaine as a reinforcer after delta-9-THC pre-exposure may be because the previous exposure to the cannabinoid agonist enhanced the aversive/anxiogenic effects of cocaine (145).
The contrasting results between species and schedules of drug delivery together suggest that the endocannabinoid system might impair other brain processes related to cocaine self administration which might in turn reduce the ability to detect the reinforcing effects of cocaine. Certainly, a better understanding and clinical prediction of the influence of the endocannabinoid system on the reinforcing effects of cocaine will come from studies of the effects of cannabinoid agonists and antagonists under a variety of procedures (including progressive-ratio schedules) and with a variety of cannabinoid exposure procedures in primates.
2.6 Effects of cannabinoids on animal models of extinction and reinstatement of cocaine-seeking behavior
One of the major obstacles to successfully achieving withdrawal or abstinence from drug use stems from the difficulty of finding effective strategies against relapse to drug use that is thought to result from craving for the abused substance. Other than behavioral therapy, potential pharmacological support might alleviate craving in abstinent individuals, thus reducing the likelihood of relapse. Although the validity of preclinical models of relapse to drug-seeking and drug-taking behaviors is still under discussion (147, 148, 149, 150), these procedures might give some useful information about which drugs and which brain areas might interfere with the processes that underlie relapse.
In the last years, as it has become increasingly clear that the endocannabinoid system plays a role in different aspects related to substance abuse, researchers have explored the involvement of the cannabinoid system in the neurobiology of relapse to drug-seeking (see citation 151 for review). In rats, when cocaine self-administration behavior was extinguished by substituting saline for cocaine injections (152), experimenter administration of increasing doses of delta-9-THC, 3 hours after withdrawal, failed to induce/reinstate cocaine-seeking behavior. However, administration of HU210, a synthetic cannabinoid agonist which has a higher affinity and greater intrinsic activity than delta-9-THC at CB1 receptors, provoked relapse to cocaine-seeking after a prolonged (14 days) withdrawal from cocaine (104). In addition to differences in treatment, the different outcome of these reports could be due to differences in abstinence strength, because in the first and second reports rats were abstinent from cocaine only for three hours (152) and 14 days (104), respectively. These different conditions might suggest time-related adaptations of the brain systems involved in cocaine-seeking behaviors (153), with no influence of the endocannabinoid system during the early stages of withdrawal from cocaine. On the other hand, when the selective antagonists of cannabinoid CB1 receptors Rimonabant and AM251 were administered alone they did not reinstate cocaine-seeking behavior (104, 154), but both drugs reduced or inhibited cocaine-seeking behavior evoked by priming injections of cocaine (104, 154). It is also interesting to note that Rimonabant reduced cocaine seeking behavior induced by cocaine associated cues, but not stress-induced reinstatement (104), suggesting the involvement of the endocannabinoid system only in some specific processes or pathways related to cocaine-seeking behavior. To this end, it is also interesting to note that AM251 did not block the DA-stimulating effects of cocaine in the NAc, but it attenuated the effects of cocaine on glutamate levels in the NAc, which might be a better neurochemical correlate of cocaine-seeking behavior (154).
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3. Human studies
Though not overlapping with the purpose of this review, the "gateway drug" hypothesis of marijuana use is an interesting example of a link or association between cocaine dependence and cannabinoid agonists. Following this hypothesis, marijuana consumption could serve as a gateway drug for subsequent use and abuse of other illicit substances in a progression that can lead towards dependence on heroin, cocaine or other psychostimulants (155, 156, 157, 158). There is still an ample debate about the factors studied and taken into consideration, and the data obtained which support this theory (see for example citations 159, and 160). It is interesting to note, however, that independently from the factor/s that link marijuana use to subsequent abuse of other illegal substances, there are also studies that show a very high prevalence of marijuana use and dependence in cocaine dependent individuals (26, 161, 162, 163). Comorbidity of cocaine dependence with other drug dependencies is also very common (25). Thus the occurrence of cannabis dependence in cocaine dependent subjects might suggest that individual vulnerability for a certain drug can be extended to other drugs (160), but also that common risk factors (genetic, environmental, etc.) might jointly influence the individual drug liability and the links between cannabis and other drug dependencies (163).
With regard to genetic risk factors, it is interesting to note that a human genetic variant of the cannabinoid receptor gene CNR1 has been associated with susceptibility of drug dependence in a non-Hispanic, Caucasian population (164). The association was stronger with intravenous drug use, and was greatest for cocaine, amphetamine, and marijuana dependence. Non-significant association data have been instead obtained in replication of the original findings by Comings and colleagues (165, 166). In a recent paper (31) these contrasting data are discussed as the result of the poor definition of the cannabinoid receptor CNR1 gene's structure, regulation and variation. In the same report, indeed, the authors have shown an improved definition of the human cannabinoid receptor CNR1 gene's locus and variants (31), and it has been also shown that haplotypes toward the 5' end of the cannabinoid receptor CNR1 gene's exons and introns differentiate between non-drug users and drug-abusers of European-American, African-American, and Japanese subjects. Also, a recent report by Ballon and colleagues, 2006 (167), supports the association of the cannabinoid receptor CNR1 gene polymorphism with predisposition to cocaine dependence in an African-Caribbean population.
Another interesting association, from a therapeutic perspective, has been evaluated studying the relationship between marijuana use during cocaine abstinence and relapse to cocaine use. In a study by Labigalini et al., 1999 (168), the authors describe a clinical observation based on spontaneous accounts by Brazilian cocaine-crack abusers, undergoing their first psychiatric assessment, reporting use of cannabis in order to get relief from cocaine-withdrawal symptoms. From a sample of 25 subjects, 68 percent ceased to use crack-cocaine during the study, which lasted 9 months. The small sample and the short period of time suggest a cautious approach to the results outcome; further studies are warranted. A more recent study (169) in New York used a much larger sample of cocaine addicts (144 subjects), and examined whether cannabis use after discharge from inpatient treatment could affect relapse to cocaine use. Data from this study showed that about a third of the subjects used cannabis after discharge and that cannabis use substantially increased the risk of relapse to cocaine use, significantly reduced the achievement of sustained remission, and significantly increased relapse to cocaine use after sustained remission (169). In agreement with this latter report is a preclinical investigation (described above, section 2.6) showing cannabinoid-induced reinstatement of cocaine seeking behavior in rodents (104).
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4. Summary and concluding remarks

Most of the scientific articles reviewed in the present manuscript have described studies of cannabinoid CB1 receptor agonists and antagonists tested against cocaine effects in preclinical models thought to be predictive of cocaine abuse. These studies have provided interesting results, especially for the ability of cannabinoid antagonists, and Rimonabant in particular, to significantly counteract some of the reinforcing actions of cocaine (104, 143). Collectively, the studies suggest that a cannabinoid tone, impaired by cannabinoid antagonist administration, is indeed involved in many of the reinforcing effects of cocaine which are believed to be responsible for cocaine abuse and addiction. On the other hand, there are no studies available showing interactions of drugs acting as cannabinoid levels modulators/enhancers on cocaine-induced behaviors. These drugs affecting directly the endogenous cannabinoid tone could interfere with cocaine effects in these preclinical procedures and could substantially increase our knowledge about the cannabinoid-neurobiology related to cocaine dependence.

Suggestions about possible genetic predisposition/vulnerability to cocaine dependence from human studies due to variants of the cannabinoid receptor CNR1 gene have given more strength to the link between endocannabinoids and cocaine. Due to the widespread distribution of cannabinoid receptors in the brain, and their abundance in brain areas playing pivotal roles in drug abuse and addiction, the different expression and regulation of cannabinoid receptors induced by genetic differences (31) might be an important factor in the predisposition or vulnerability to drug dependence. For this reason, the potential to directly interact with endocannabinoid tone in selected brain areas, an effect that can be obtained with endocannabinoid uptake inhibitors or metabolism blockers (4, 60, 62)(as shown also in genetically modified mice, 170), as compared to widespread actions of cannabinoid receptors agonists/antagonists, should be one of the next challenges in the research for medications able to counteract the abuse- and dependence-related behavioral/neurobiological effects of cocaine.

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Footnotes

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Cocaine-induced endocannabinoid release modulates behavioral and neurochemical sensitization in mice

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Associated Data

Supplementary Materials

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Abstract

The endocannabinoid system has been implicated in the development of synaptic plasticity induced by several drugs abused by humans, including cocaine. However, there remains some debate about the involvement of cannabinoid receptors/ligands in cocaine-induced plasticity and corresponding behavioral actions. Here we show that a single cocaine injection in Swiss-Webster mice produces behavioral and neurochemical alterations that are under the control of the endocannabinoid system. This plasticity may be the initial basis for changes in brain processes leading from recreational use of cocaine to its abuse and ultimately to dependence. Locomotor activity was monitored with photo-beam cell detectors, and accumbens shell/core microdialysate DA levels were monitored by HPLC with electrochemical detection. Development of single-trial cocaine-induced behavioral sensitization, measured as increased distance traveled in sensitized mice compared to control mice, was paralleled by a larger stimulation of extracellular dopamine (DA) levels in the core but not the shell of the nucleus accumbens. Both the behavioral and neurochemical effects were reversed by CB1 receptor blockade produced by rimonabant pretreatments. Further, both behavioral and neurochemical cocaine sensitization were facilitated by pharmacological blockade of endocannabinoid metabolism, achieved by inhibiting the fatty acid amide hydrolase enzyme. In conclusion, our results suggest that a single unconditioned exposure to cocaine produces sensitization through neuronal alterations that require regionally specific release of endocannabinoids. Further, the present results suggest that endocannabinoids play a primary role from the earliest stage of cocaine use, mediating the inception of long-term brain-adaptive responses, shaping central pathways, and likely increasing vulnerability to stimulant abuse disorders.

Keywords: Behavioral sensitization, Cannabinoid CB1 receptors, Cocaine addiction, Endocannabinoids, In-vivo Dopamine microdialysis, Nucleus Accumbens

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Introduction

Repeated psychostimulant administration (Cadoni et al., 2000; Kalivas and Duffy, 1993), as well as even a single exposure to a psychostimulant, can produce long-lasting sensitization to acute behavioral stimulant effects in rodents (Saal et al., 2003; Ungless et al., 2001). Long-term neurobiological adaptations such as increasing strengths of excitatory synapses in midbrain dopaminergic regions are thought to play an important role in various sensitized behavioral effects, including those related to the development of drug craving and dependence (Bowers et al., 2010; Kauer and Malenka, 2007; Pierce and Vanderschuren, 2010; Robinson and Berridge, 1993). Among the many neurotransmitter systems implicated in drug abuse and dependence, recent evidence has suggested the involvement of the endocannabinoid system in stimulant sensitization, even though there is still a debate about its precise role as well as for its potential effects related to stimulant abuse and dependence (as reviewed by Arnold, 2005; Tanda, 2007; Wiskerke et al., 2008). For instance, genetic deletion of cannabinoid CB1 receptors in mice (Soria et al., 2005) or pharmacological blockade of CB1 receptors decreases the reinforcing effects of cocaine (Soria et al, 2005; but see also Cossu et al., 2001), cocaine-induced stimulation of nucleus accumbens (NAC) DA concentrations measured by microdialysis or voltammetry (Cheer et al., 2007; Li et al., 2009; but see Xi et al., 2006), and the development of cocaine-induced neurobiological adaptations (Corbille et al., 2007; Filip et al., 2006; Gerdeman et al., 2008; but see Lesscher et al., 2005).

Previous studies have reported that psychostimulants, such as cocaine or amphetamine, as well as dopamine D1 or D2-receptor activation may induce DA-dependent release of endocannabinoids in selected brain regions (Centonze et al., 2004; Ferrer et al., 2003; Giuffrida et al., 1999; Thiemann et al., 2008). Thus, endocannabinoids, acting as retrograde synaptic messengers (Wilson and Nicoll, 2002), may be involved in functional effects of cocaine, including those related to synaptic plasticity. Moreover, an increase in endocannabinoid levels might facilitate immediate, sub-second, acute effects of cocaine (Cheer et al., 2007). Others however have not found increased levels of the endocannabinoid, anandamide, in several mesocorticolimbic regions after chronic cocaine administration in rats (Caille et al., 2007; Gonzalez et al., 2002).

In the present study we tested the hypothesis that CB1 receptors, activated by cocaine-induced endocannabinoid release in mice (Centonze et al., 2004), might be involved in the very early stages of neuronal adaptations underlying development of behavioral sensitization. To focus on early stages of the process, the effects of a single cocaine injection was assessed. Under the same experimental conditions we also investigated how enhancement of endocannabinoid release, by blockade of endocannabinoid metabolic enzymatic pathways, might influence the development of behavioral sensitization when cocaine is administered at sub-optimal doses. Finally, the NAC shell and core are main terminal areas of mesolimbic DA neurons and have documented critical roles in cocaine reinforcement (Di Chiara, 2002; Koob and Volkow, 2010) that are involved from the

initial exposure to drug (Di Chiara et al., 1999; Di Chiara et al., 1998; Pontieri et al., 1995). Therefore, we also investigated the involvement of these areas in cocaine sensitization and the involvement of the endocannabinoid system in modulating DA activity in these regions.

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Materials and Methods

Subjects

Male Swiss-Webster mice (Taconic, Germantown, NY), weighing 30-40 g, were housed in groups of four per cage in a large temperature and humidity controlled colony room, under a 12-h light/dark cycle (lights on at 7:00 a.m.). Mice had free access to food and water, except during the behavioral and neurochemical tests.

Drugs and Injections

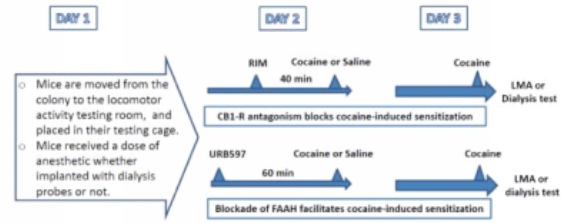
The CB1 receptor antagonist, rimonabant (SR 141716A, abbreviated as RIM in the figures, N-(piperidin-1-yl)-5-(a-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl1-1H-pyrazole-3-carboxyamide HCl, NIDA Drug Supply Program) was dissolved in a vehicle comprised of 2% Tween 80, 2% alcohol, and saline. Cocaine HCl (Sigma Chemicals, St. Louis, MO or NIDA Drug Supply Program) was dissolved in saline (0.9% NaCl). URB 597 was provided by Dr. Daniele Piomelli, Department of Pharmacology, University of California, Irvine, CA, USA. It was dissolved in a vehicle comprised of 2% Tween 80, 2% alcohol, and saline. All injections were delivered i.p. (0.1ml/10g.), and the respective vehicles served as control injections. Drug doses and pretreatment times are from a preliminary study presented in abstract form (Tronci and Tanda, 2007).

Study Design

The main dependent measure was the amount of locomotor activity or the change in dopamine concentration induced by cocaine on Day 3 after various sensitizing treatments on Day 2. On Day 1 all mice in all groups (behavioral and microdialysis) were moved from the animal vivarium to the testing rooms, and after about 30 minutes were anesthetized and placed in their testing cages, which housed them for the remainder of the study (see Table 1). Some control groups were administered saline on Day 1 as a control for the injection of anesthetic in other groups. No significant differences in cocaine-induced sensitization in locomotor activity measures were found between the anaesthetized and saline control groups, and for that reason the data from groups receiving saline on Day 1 have been included with data from the corresponding groups that received anesthesia on Day 1.

Table 1

Timeline of treatments for behavioral and neurochemical tests


DAY 1
DAY 2
DAY 3
Mice are moved from the colony to the locomotor activity testing room, and placed in their testing cage.
Mice received a dose of anesthetic whether implanted with dialysis probes or not.
RIM
Cocaine or Saline
40 min
Cocaine
LMA or Dialysis test
CB1-R antagonism blocks cocaine-induced sensitization
URB597
Cocaine or Saline
60 min
Cocaine
LMA or dialysis test
Blockade of FAAH facilitates cocaine-induced sensitization

Day 2 vehicle or cocaine was injected following either rimonabant or URB 597 treatment. Injections on Day 2 were administered 24 hr before test doses of cocaine on Day 3. All injections and testing were conducted during the light period (lights on at 7:00 am and off at 7:00 pm) of the light-dark cycle. All behavioral and microdialysis tests were performed on Day 3.

Behavioral Experiments

Horizontal locomotor activity (expressed as distance traveled, cm) was measured using monitors (Omnitech Electronics Inc., Columbus, OH) with a horizontal matrix of 16 photo-beams, fixed at a height of 2 cm, and spaced at 5-cm intervals along the interior perimeter. Eight beams per side crossed the test chamber (transparent acrylic box, 40×40×30 cm high), which was located within the monitor.

1) Effects of cannabinoid CB1 receptor blockade on development of single-trial behavioral sensitization to cocaine

On Day 2, between 20 and 22 hr after their transfer to the testing room and placement in the testing cage, mice were divided into groups that were injected with either vehicle or rimonabant (0.3, 1.0, or 3.0 mg/kg i.p.). Each of these four groups was further divided into two groups and injected 40 min later with saline or cocaine (20 mg/kg). On Day 3 (24 hr after the cocaine or saline injections) each group was further divided into groups (n=6) that were injected with cocaine (10 or 20 mg/kg). Immediately after this last injection, locomotor activity measured by distance traveled was monitored for 60 min. Thus, this experiment assessed the development of behavioral sensitization 24 hr after a single cocaine dose, and the potential of increasing doses of rimonabant to antagonize the sensitization.

2) Effects of FAAH blockade on development of cocaine-induced behavioral sensitization

On Day 2, between 20 and 22 hr after their transfer to the testing room and placement in the testing cage, mice were divided into groups that were injected with either vehicle or URB 597 (1.0 or 3.0 mg/kg i.p.). Each of these three groups was further divided into two groups and injected 60 min later with saline or cocaine (10 mg/kg). On Day 3 (24 hr after the cocaine or saline injections) subjects in each group (n=6) were injected with cocaine (20 mg/kg). Immediately after this last injection locomotor activity was monitored for 60 min. Thus, this experiment assessed the development of behavioral sensitization 24 hr after a combination of URB 597 and a cocaine dose that was insufficient to produce significant behavioral sensitization in order to assess the potential of URB 597 to potentiate the development of cocaine sensitization.

Statistical Analysis

Locomotor activity data were expressed as the average distance traveled, cm ±SEM, obtained for each experimental group of animals during the first 30 min after injection. Results are expressed as group means (±SEM). Data were analyzed by one- or two-way ANOVA with treatment and pre-treatment as factors. Significant changes were subjected to Bonferroni's or Tukey's post-hoc tests. Significant differences from control groups, $p<0.05$, are represented in the figures with a * or # mark. All results of statistical analyses are shown in the figure legends in order to make the text clear and easy to follow.

Microdialysis Experiments

Concentric dialysis probes were prepared with AN69 fibers (Hospal Dasco, Bologna, Italy) as described previously (Loland et al., 2012; Tanda et al., 2009). Briefly, two 4-cm pieces of silica-fused capillary tubes (the inlet and outlet tubing of the probes) were inserted into a 4-mm capillary dialyzing fiber (closed by a drop of glue on the other side), with the inlet tubing set at about 0.1 mm from the closed, glued end of the fiber and the outlet set at 1mm from the inlet tip. Note that the glue tip was cone-shaped so that implanting the probe with a continuous infusion would produce minimal damage to the surrounding tissue. The open end of the dialysis membrane was then glued and the protruding two silica-fused pieces of tubing were inserted and glued into a 22-gauge stainless steel needle (1.7-mm length). The needle was then clipped to a CMA/10 clip (CMA Microdialysis AB, Solna, Sweden) and mounted in a stereotaxic holder. The exposed dialyzing surface of the fiber (i.e., the portion not covered by glue) was limited to

the lowest 1.0 mm portion of the probe.

Surgery

On Day 1 mice were anesthetized under a mixture of Xylazine (10 mg/kg, i.p.) and ketamine (60 mg/kg, i.p.) and a concentric dialysis probe was implanted (see table 1). Mice were placed in a stereotaxic apparatus, the skull was exposed, and a small hole was drilled to expose the dura. Mice were then randomly implanted in the right or left side with a concentric dialysis probe aimed at the NAC shell or core (shell: A= +1.5, L= ±0.6, V= -5.1; core: A= +1.3, L= ±1.3, V= -4.9; anterior, A, lateral, L, vertical, V, in millimeters from bregma), according to the mouse brain atlas by Paxinos and Franklin (2001). Figure 1 shows drawings of forebrain sections with the boundaries (superimposed rectangles) within which microdialysis probe tracks were considered correct probe placements. After surgery, mice were allowed to recover in square Plexiglas cages with bedding on the floor, that were equipped with overhead quartz-lined fluid swivels (Instech Laboratories Inc., Plymouth Meeting, PA) for connections to the dialysis probes.

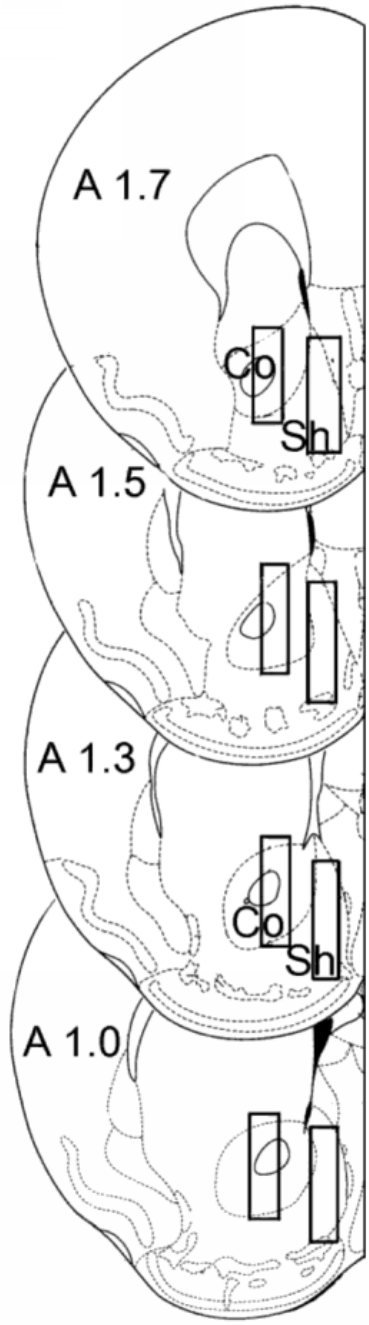


Figure 1

Brain microdialysis probe placements in the NAC shell or core. Forebrain sections, redrawn from Paxinos and Franklin (2001), showing the limits of the positions of the dialyzing portions of the microdialysis probes (superimposed rectangles). On each section the anterior coordinate (measured from bregma) is indicated.

Effects of cannabinoid CB1 receptor blockade on cocaine-induced stimulation of DA extracellular levels in sensitized and non-sensitized mice

On Day 2, treatments were administered starting about 18-20 hr after probe implant (see table 1). Mice were divided into different groups that were injected with either vehicle or rimonabant (1.0 or 3.0 mg/kg i.p.). Each of these groups was further divided into two groups and injected 40 min later with saline or cocaine (20 mg/kg). Microdialysis sampling was initiated on Day 3 (approximately 22-22.5 hr after Day 2 treatments). Microdialysis probes were perfused with Ringer's solution (147.0 mM NaCl, 2.2 mM CaCl2, and 4.0 mM KCl), delivered by a 1.0-ml syringe and operated by a BAS Bee Syringe Pump Controller (BAS Bioanalytical Systems, West Lafayette, IN), at a constant flow rate of 1 μl/min. Collection of dialysate samples (10 μl) started after 30 min, and samples were taken every 10 min and immediately analyzed, as detailed below. Cocaine (20 mg/kg) was injected on Day 3 when stable DA values (less than 10% variability) were obtained for at least three consecutive samples (typically after about 1 hr). Dialysis sampling continued once every 10 min for 2 hr.

Effects of inhibition of the FAAH enzyme on cocaine-induced stimulation of DA extracellular levels in the NAC core in sensitized and non-sensitized mice

As above, on Day 2 treatments were administered starting about 18-20 hr after probe implant. Mice were divided into different groups that were injected with either vehicle or URB 597 (1 or 3.0 mg/kg i.p.), and one hr later with saline or cocaine (10 mg/kg i.p.). The URB 597 experiment was carried out only from NAC core dialysates, as no instances of cocaine-induced sensitization to changes in DA levels were previously found in the NAC shell. Microdialysis sampling was initiated on Day 3 as described above.

Analytical Procedure

Dialysate samples (10 μl) were injected without purification into a high-performance liquid chromatographyapparatus equipped with a MD 150- x 3.2-mm column, particle size 3.0 μm (ESA, Chelmsford, MA) and a coulometric detector (5200a Coulochem II or coulochem III; ESA Inc., Chelmsford, MA) to quantify DA. The oxidation and reduction electrodes of the analytical cell (5014B; ESA Inc.) were set at +125 and −175 mV, respectively. The mobile phase, containing 100 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.5 mM Na-octyl sulfate, and 18% (v/v) methanol (pH adjusted to 5.5 with Na_2HPO_4) was pumped by an ESA 582 (ESA Inc.) solvent delivery module at 0.50 ml/min. Assay sensitivity for DA was 2 fmol per sample.

Histology

Histology was performed in accordance with previous studies (Tanda et al., 2009). Brains were cut on a vibratome in serial coronal slices oriented according to the atlas by Paxinos and Franklin (Paxinos and Franklin, 2001) to identify the location of the probes. Only the experiments in which the probes were appropriately located inside the NAC shell or core boundaries have been considered and used for the DA microdialysis results shown in the present study (see Fig. 1).

Statistical Analysis

Results were expressed as a percentage of basal DA values, calculated as the mean of the last 3-4 consecutive samples (differing no more than 15%) immediately preceding the first drug or vehicle injection. All results are presented as group means (±SEM). Differences in basal levels of DA between different experimental groups within the same brain area, or between different brain areas, were analyzed by one-way ANOVA. Statistical analyses of experimental data were carried out with Statistica-6 software using a two-way ANOVA (drug dose and time as factors) or three-way ANOVA (drug dose for pretreatment, drug dose for treatment, and time) for repeated measures over time, with results from treatments showing overall changes subjected to post hoc Tukey's test. Results were considered significant at $p<0.05$. All results of statistical analyses are shown in the figure legends in order to make the text clear and easy to follow.

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Results

Mice previously treated 24 hr prior with cocaine (20 mg/kg) displayed a robust and significant increase in distance traveled after the second injection of cocaine (10 or 20 mg/kg) compared to those treated 24 hr prior with saline ($p<0.05$; Fig. 2A1 and 2B1; compare white to black bars).

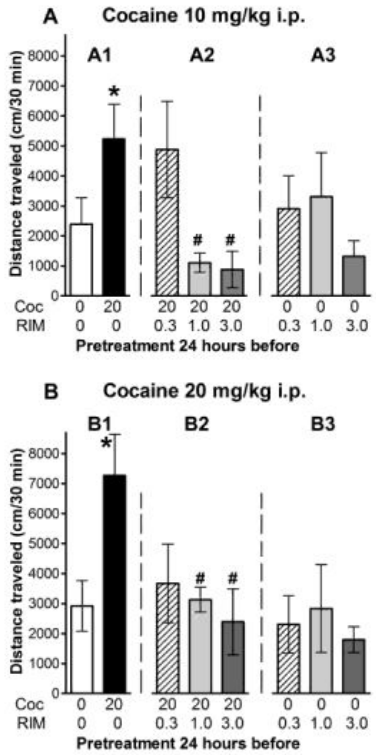

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Figure 2

Cocaine-induced single trial behavioral sensitization is blunted by antagonism at CB1 receptors

Locomotor activity response to an acute challenge with cocaine (panel A, 10 mg/kg, or panel B, 20 mg/kg) in mice pretreated 24 hr and 40 minutes before the cocaine challenge with vehicle [A two-Way ANOVA yielded significant main effects of cocaine pre-exposure, $F(1,67) = 11.235$, $p<0.002$, and cocaine treatment, $F(2,67) = 7.527$, $p<0.002$, and a non-significant effect of their interaction: $F(2,67) = 2.682$, $p=0.075$] or increasing doses of rimonabant (RIM), 0.3 (hatched bars), 1.0 (light grey filled bars), and 3 mg/kg i.p., (dark grey filled bars), followed 40 minutes later with either saline (unfilled bars) or cocaine 20 mg/kg i.p. (solid black bars). [One-Way ANOVA of the effects of RIM 0.3-3.0 mg/kg pretreatments was significant at both the cocaine challenges, 10 mg/kg $F(3,25) = 3.689$, $p<0.005$), and 20 mg/kg $F(3,32) = 3.042$, $p<0.005$]. The results are expressed as the average distance traveled (cm/30 min) ± S.E.M. obtained for each experimental group of animals during the first 30 minutes after injection. Significant differences from control groups,* = $p< 0.05$ VS saline, or # = $p<0.05$ VS cocaine.

Increasing doses of the CB1 receptor antagonist, rimonabant (0.3 to 3.0 mg/kg), administered 40 minutes before the otherwise sensitizing dose of cocaine (20 mg/kg, i.p.) on Day 2, dose-dependently ($p<0.05$) attenuated (Fig. 2A2 and 2B2) the sensitized response to cocaine (10 or 20 mg/kg i.p.). In mice that did not receive a sensitizing dose of cocaine (Fig. 2A3 and 2B3), rimonabant administration, 40 minutes before saline pretreatments on Day 2, did not significantly modify the stimulation of locomotor activity produced by either 10 ($p>0.05$), or 20 ($p>0.05$) mg/kg of cocaine administered 24 h later. As shown in the supplementary information (suppl. Fig. 1), rimonabant pretreatment failed to significantly modify the non-

sensitized stimulation of locomotor activity induced by a single acute i.p. injection of 10 or 20 mg/kg of cocaine ($p>0.05$). Thus, it appears that under the present experimental conditions rimonabant affects the sensitized response to cocaine but, across the same range of doses, not its acute stimulatory effects. In general the effects of rimonabant were statistically significant, although there was an insignificant trend for rimonabant 0.3 mg/kg to attenuate the ambulatory activity induced by 20 mg/kg of cocaine more than that induced by 10 mg/kg group (see Fig. 2, A2 and B2).

In vivo microdialysis studies of DA levels in the NAC core indicated that pre-exposure to cocaine (on Day 2) increased the response to cocaine when administered 24 hr later (Fig. 3A and B, compare filled and open circles in both panels). The stimulated levels of NAC core DA produced by cocaine (at either 10 or 20 mg/kg, i.p.) were greater in mice pre-exposed to cocaine (20 mg/kg i.p., 24 hr before) than those pre-exposed to saline under the same experimental conditions ($p<0.05$).

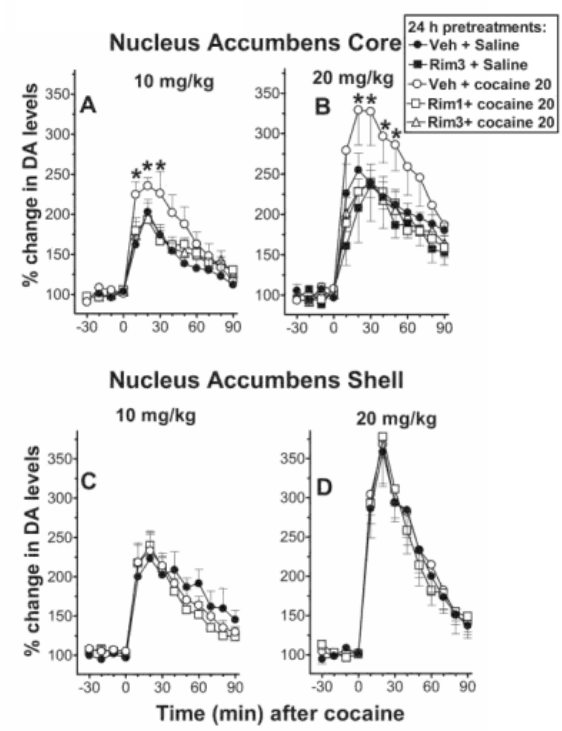

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Figure 3

Cocaine-induced single trial behavioral sensitization is accompanied by increased stimulation of NAC core, but not NAC shell, DA transmission, which is blocked by antagonism at CB1 receptors

The figure shows the time course of the effects of systemic administration of cocaine 10 mg/kg (top panels, A, C), or 20 mg/kg (lower panels, B,D) on extracellular levels of DA in dialysates from the NAC core (left panels) or shell (right panels) in control animals (● filled circles, number of mice and basal levels of extracellular DA expressed as femtomoles/10-μl sample ± S.E.M. were: n=5, 41.1 ± 3.9, and n=5, 42.9 ± 4.9 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the core; n=6, 57.9 ± 6.7 fmoles/sample, and n=9, 48.4 ± 4.7 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the shell), or in animals sensitized to cocaine (○ unfilled circles, n=7, 37.4 ± 2.0 fmoles/sample, and n=6, 30 ± 3.4 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the core; n=9, 48.5 ± 3.0 fmoles/sample, and n=8, 51.3 ± 4.2 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the shell), in animals pretreated with RIM 1 mg/kg and cocaine 20 mg/kg (□ unfilled squares, n=6, 52.4 ± 3.6 fmoles/sample, and n=6, 51.8 ± 4.4 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the core; n=9, 44.2 ± 3.7 fmoles/sample, and n=6, 29.1 ± 3.1 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the shell) and in animals pretreated with RIM 3 mg/kg and cocaine (▵, unfilled triangles, n=6, 59.1 ± 5.7 fmoles/sample, and n=6, 52.1 ± 9.1 fmoles/sample, for cocaine 10 and 20 mg/kg, respectively in the core) or pretreated with RIM 3 mg/kg and saline (▌filled squares, n=4, 35.0 ± 7.7 fmoles/sample, for cocaine 20 mg/kg in the core) 24 hr before microdialysis tests. [Three-Way ANOVA (RIM pretreatment X cocaine treatment X time) showed in the core significant main effects of cocaine dose $F(1,42)= 15.296$, $p<0.001$, RIM pretreatment $F(3, 42) = 3.168$, $p<0.05$, and time $F(6,252)= 91.066$, $p<0.001$; also, there were significant interactions of cocaine dose × time $F(18,252) = 19.735$, $p<0.001$, RIM pretreatment × time $F(18,252)= 2.297$, $p<0.05$; non-significant interactions of RIM pretreatment × cocaine dose $F(3.42)=0.518$, NS; and pretreatment × cocaine dose × time interaction $F= (18,252)= 0.486$, NS. Three-Way ANOVA in the shell showed significant main effects of cocaine dose $F(1,36)= 24.057$, $p<0.001$, and time $F(12,432)= 101.088$, $p<0.001$, and a non-significant main effect of RIM pretreatment $F(2, 36) = 0.139$; also, there were significant interactions of cocaine dose × time $F(12,432) = 19.735$, $p<0.001$, non-significant interactions of pretreatment × cocaine dose $F(2,36)=0.872$; pretreatment × time $F(24,432)= 1.007$; and pretreatment × cocaine dose × time interaction $F= (24,432)= 0.594$]. Results are means with vertical bars representing S.E.M. of the amount of DA in 10 minutes dialysate samples, expressed as percentage of basal values.

The sensitized DA stimulation obtained in the NAC core was reduced to saline-pretreatment control levels when mice received rimonabant (1 or 3 mg/kg, i.p.) on Day 2 40 min before the sensitizing dose of cocaine (Fig. 3A and B, compare open squares or triangles to open circles in both panels). As with locomotor activity, rimonabant did not significantly alter the effects of acute administration of cocaine on stimulation of DA levels in the NAC core (Suppl. Fig. 2) in mice that were not sensitized by prior cocaine treatment ($p>0.05$).

A sensitizing dose of cocaine (20 mg/kg, ip) on Day 2 did not significantly alter the stimulation of DA levels in the NAC shell on Day 3 induced by either 10 or 20 mg/kg cocaine ($p>0.05$) (Fig. 3, compare filled and open circles in panels C and D). Further, no substantial change ($p>0.05$) in the dopaminergic response to cocaine in the NAC shell was produced by rimonabant pretreatment (1.0 mg/kg, i.p.) 40 min before cocaine injection on Day 2 (Fig. 3; compare open squares to other points in panels C and D). That dose of rimonabant decreased the cocaine-induced sensitized locomotor response and dopaminergic response in NAC core. Also, as shown in the supplementary information, rimonabant administered 40 minutes before cocaine (10 or 20 mg/kg, i.p.) failed to significantly change the stimulation of DA levels in the NAC shell after the acute injection of cocaine ($p>0.05$) (Suppl. Fig.2).

The blockade of behavioral and neurochemical cocaine sensitization by rimonabant suggests that the sensitization requires activation of CB1 receptors, which can be the result of cocaine-induced on-demand release of endocannabinoids (Centonze et al., 2004; Cheer et al., 2007). That hypothesis was tested by pretreating a group of mice with a lower dose of cocaine (10 mg/kg i.p.), which by itself did not induce behavioral sensitization (Fig. 4) possibly because it did not release endocannabinoids at levels sufficient to activate CB1 receptors. The hypothesis predicts that the effects of a less than fully efficacious dose of cocaine will be enhanced by blocking endocannabinoid metabolism (Solinas et al., 2006; Solinas et al., 2010).

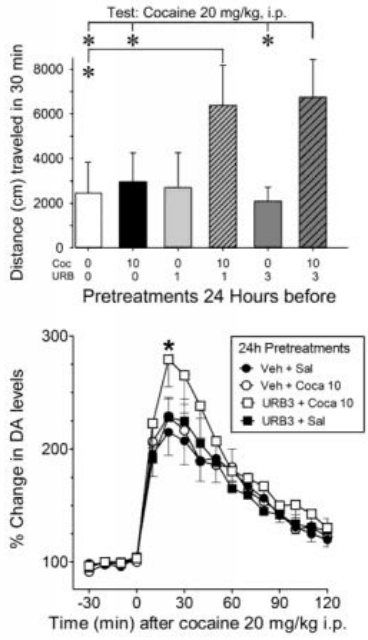

Figure 4

Blockade of the FAAH enzyme by URB597 facilitates the occurrence of cocaine-induced single trial behavioral and NAC core neurochemical sensitization in animals pretreated with a non-sensitizing dose of cocaine

This figure shows on panel A that mice pretreated with a 10 mg/kg dose of cocaine 24 hr in advance, and then tested with a dose of cocaine, 20 mg/kg i.p., did not display behavioral sensitization, measured as increased distance traveled, as compared with mice that were pretreated with saline 24 hr before [one way ANOVA shows NS effect of sensitization treatment, $F(1,14)=0.029$, $p=0.866$]. However, increased locomotor activity, indicating behavioral sensitization, was observed in mice tested with cocaine 20 mg/kg i.p. when they received a 24 hr pretreatment with cocaine 10 mg/kg that was preceded by administration 1 hr pretreatment with either 1 or 3 mg/kg i.p. doses of URB597 (which are known to block the FAAH) [two way ANOVA shows significant main effects of URB pretreatment, $F(2,52)=3.326$, $p<0.05$, cocaine treatment, $F(1,52)=7.586$, $p<0.01$, and their interaction, $F(2,52)=3.163$, $p<0.05$]. Panel B shows dopamine microdialysis data from mice implanted with probes in the NAC core that, in agreement with locomotor activity data, display a larger DAergic response in this brain area, indicating sensitization, obtained in animals pretreated with the FAAH blocker, URB597 (URB) 3 mg/kg i.p. administered 1 hr before a non-sensitizing dose of cocaine, 24 hr before the testing dose of cocaine, 20 mg/kg i.p., compared to animals that received URB vehicle pretretments (pretreatment groups' number of animals, n, and basal DA values, fmoles/sample ± S.E.M., were: Veh+Sal, ● filled circles, n=4, 63.18±8.6; Veh+cocaine 10 mg/kg, ○ unfilled circles, n=8, 53.2+5.0; URB+Sal, ▌filled squares, n=5, 64.7±3.9; URB+cocaine 10 mg/kg, □ unfilled squares, n=6, 52.4±7.9) [two way ANOVA for repeated measures shows significant main effects of URB pretreatment, $F(1,27)=4.556$, $p<0.05$, and time, $F(3,81)=76.781$, $p<0.001$, and significant URB pretreatment by time interaction, $F(3,81)=2.847$, $p<0.05$]. In microdialysis experiments, results are expressed as means with vertical bars representing S.E.M. of the amount of DA in 10 minutes dialysate samples, expressed as percentage of basal values. * = $p< 0.05$, Tukey's post-hoc test.

To test this hypothesis a group of mice received on Day 2 the lower (10 mg/kg i.p.) dose of cocaine 60 min after pretreatment with a fatty acid amide hydrolase (FAAH) inhibitor, URB597 (1 and 3 mg/kg, i.p.), which blocks the metabolism of the endocannabinoid, anandamide (Kathuria et al., 2003). In these mice, 10 mg/kg cocaine as expected, did not sensitize subjects to a 20 mg/kg dose of cocaine administered on Day 3 (Figure 4A, compare white and black bars). URB5897 when administered before vehicle on Day 2 at either 1.0 or 3.0 mg/kg did not change the response to cocaine on Day 3 (Fig. 4A, gray bars). However, URB597 at both 1.0 and 3.0 mg/kg enhanced the effect of the 10 mg/kg dose of cocaine on Day 2 such that sensitization was evident on Day 3 (Fig. 4, stippled bars). The effect of the challenge dose of cocaine (20 mg/kg) in mice that received URB597 with 10 mg/kg of cocaine was substantially and significantly greater than that produced when that dose of cocaine was preceded by vehicle injection (Fig. 4, top panel).

URB597 administered 60 min before cocaine (10 mg/kg, i.p.) on Day 2 also enhanced the effects on DA levels of 20 mg/kg of cocaine administered on Day 3 (Fig. 4B, compare open squares to filled circles). As expected, 10 mg/kg of cocaine 60 min after a vehicle injection on Day 2, did not sensitize subjects to a Day 3 injection of 20 mg/kg of cocaine (Fig. 4B, compare open and filled circles). Similarly, URB597 administered on Day 2 with a vehicle injection 60 min later had no effect on the response to 20 mg/kg of cocaine on Day 3 (Fig. 4B, compare filled squares and circles).

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Discussion

The results in the present study show that a context-independent behavioral sensitization that occurs in mice after a single exposure to cocaine (Ungless et al., 2001) can be abolished by the CB1 receptor antagonist, rimonabant. Our results also indicate that a similar neurochemical sensitization, likely related to the behavioral sensitization, occurs in the core but not in the shell of the NAC. Such sensitization in DA response in the NAC core is also attenuated by blockade of CB1 receptors with rimonabant. Finally, the cocaine-sensitizing behavioral and neurochemical effects were replicated with a small, non-sensitizing dose of cocaine under conditions in which the main metabolizing route for the endocannabinoid anandamide, FAAH, has been blocked by administering the FAAH-inhibitor URB597 (Solinas et al., 2006). It is interesting to notice that in agreement with previous studies in rats (Cadoni et al., 2000; Pontieri et al., 1995; Tanda et al., 2005) this study in mice shows a significant, larger stimulation of DA levels in the shell as compared to the core after acute cocaine administration. Also in agreement with Cadoni et al. (2000), such significance is lost after cocaine-induced sensitization. The differences in DA response in the NAC shell and core with acute cocaine appear at variance with those by Zocchi et al. (2003). In that report subjects were CD1 mice, implanted with a large guide cannulae less than 24 hours before the tests, compared to our Swiss-Webster mice implanted about 42-46 hours before test with a thin, likely more biocompatible probe (no guide cannulae, no metal, and only the dialyzing membrane with silica fused tubing in contact with brain tissue). Zocchi et al. (2003) reported that cocaine significantly increased the lower basal levels of DA in the NAC shell more than in the core. However, when basal variability was used as a covariate of cocaine effects, the effects in core and shell were not statistically different. In our study there were no significant differences in DA levels in the two NAC subregions and the acute effects of cocaine were greater in the shell than core. The several methodological differences between the present study and that of Zocchi et al. (2003) make the resolution of the differences impossible without further studies.

Conflicting results have been reported by several research groups about the involvement of cannabinoid receptors in the development of cocaine-induced sensitization. Compared to previous reports, the present study focused on the earliest stages of cocaine-induced sensitization, using a one-day

sensitization protocol (Ungless et al., 2001). Moreover, using a single exposure minimized complications due to conditioning/contingency effects of cocaine administration (Cossu et al., 2001; Gerdeman et al., 2008; Lesscher et al., 2005).

As shown in rats (Cadoni et al., 2000), we also found that cocaine behavioral sensitization was paralleled by a selective neurochemical sensitization of the DA response in the NAC core as compared to the NAC shell in mice. In this regard, it has been shown that cocaine-induced behavioral sensitization in rats is accompanied by structural plasticity in the core, but not in the shell, of the NAC (Li et al., 2004). In agreement with these reports, our data clearly show that behavioral sensitization in mice was accompanied by a larger neurochemical stimulation in the NAC core as compared to the shell. It is interesting to notice that interactions between endocannabinoids, cannabinoid CB1 receptors, and DA D1-D2 receptors have been recently demonstrated to enhance firing of NAC core neurons (Seif et al., 2011), even though 2-AG more than anandamide has been involved in such interaction. Under conditions of FAAH inhibition an involvement of 2-AG seems unlikely (Caprioli et al., 2012; Wiskerke et al., 2012) in the present experiments, but its involvement cannot be ruled out completely. Interactions between activation of DA D2 receptors, endocannabinoid release, and cannabinoid CB1 receptor activation have been described in vitro also in the VTA (Pan et al., 2008a, b), even though in those studies there were no distinctions between midbrain DA neurons projecting to different (i.e. shell/core) DA terminal areas. DAergic terminal areas have been the target for a study showing D2-induced retrograde anandamide release being instrumental to plasticity changes in the striatum (Gerdeman et al., 2002). The authors suggest possible roles for dysfunctions of the endocannabinoid system in developmental disorders of motor control or complex habits (Gerdeman et al., 2002). In apparent contrast with our result, Eisenstein and colleagues (2009) report that animals receiving amphetamine and URB597 show reduced behavioral sensitization compared to those receiving amphetamine and vehicle. It is interesting to note that such effects occurred only from the 5th day of URB597 treatment. In the same report rimonabant did not affect amphetamine-induced sensitization, during the 8 days of administration. The different experimental conditions (drugs, animal species, number of treatments, etc.) between our study and that of Eisenstein, and the lack of significant sensitization on day 2 compared to day 1 in their report make direct comparions difficult. In another recent report (Luque-Rojas et al., 2013) blockade of either FAAH or monoacyl-glyceryl-lipase (MGL) enzymes (which selectively block the degradation of 2-AG) neither facilitated nor blocked the development of cocaine-induced sensitization. Again, differences in experimental conditions between these studies could influence the outcomes. In the study by Luque-Rojas and colleagues (2013) cocaine was administered alone or in combination with URB597 or URB602 (an MGL-blocker that enhances circulating levels of 2-AG). Their model, administering cocaine repeatedly at doses already fully capable of inducing sensitization favors the detection of antagonism rather than sensitization (Luque-Rojas et al., 2013), instead of favoring the development of cocaine-induced sensitization, with cocaine administered at non-sensitizing doses. In our model the effects of URB597 were tested acutely, conditions under which anandamide levels released by cocaine at sub-optimal doses (i.e. not able to elicit sensitization) could be enhanced by FAAH blockade. Thus only with that enhancement would sufficient concentrations of anandamide be reached to significantly activate cannabinoid CB1 receptors which we hypothesized to be involved in the earliest stages of cocaine-induced sensitization. In a different study by Forgeaud et al. (2004), using a model of cocaine sensitization similar to ours, it was found that 24 hours after the first cocaine injection the endocannabinoid long-term-depression was abolished in the NAC. Such specific alteration in the endocannabinoid system seems directly related to the occurrence of cocaine-induced sensitization, as also confirmed by Grueter et al. (2010). These reports do not contrast with our findings. In our conditions some of the delayed effects of cocaine, including those changes related to behavioral and neurochemical sensitization in the core, were reduced by CB1 blockade or enhanced by increasing the level of cannabinoids right before the first cocaine administration. All of the cannabinoid/endocannabinoid interactions with cocaine sensitization that we have tested could counteract or facilitate its development, 24 hours later. Thus, compared to Forgeaud et al. (2004) we did not test the status or the effects of the endocannabinoid system 24 hours after the administration of cocaine. Indeed, we focused our attention on the endocannabinoid system interaction with the development and not to the expression of cocaine-induced sensitization, though expression of sensitization could be an interesting focus of future research. We want to point out that URB597 and rimonabant facilitation or blockade of sensitization was not influenced by any long-lasting effects of these drugs, as we have shown that when administered alone the drugs produced no significant change in the effects of cocaine administered 24 hours later.

Because OEA and PEA along with anandamide are substrates of the FAAH enzyme, URB597 would be expected to increase circulating levels of each. As anandamide, OEA and PEA are also endogenous ligands for the nuclear receptor PPARα, an effect mediated by that mechanism might be an alternative explanation for the observed effects of URB 597. However, URB 597 administered before saline did not produce a significant effect on sensitization in the present study. A recent report showed effects of cocaine on medium-spiny neurons in the NAC shell counteracted by administration of URB597 (Luchicchi et al., 2010), an effect not blocked by CB1 antagonists, and likely dependent from a PPARα activation. PPARα receptors and URB597 however did not appear to play a role in cocaine induced firing of VTA DA neurons (Luchicchi et al., 2010) and in cocaine-induced sensitization (Fernandez-Espejo et al., 2009). In our experiments the effects of URB 597 on cocaine-induced sensitization in the NAC core rather than the shell was tested, as significant sensitization of the effects of cocaine was only found in the former brain area.

Anandamide has been suggested to be endogenous ligand of TRPV1 receptors (See for review: Toth et al., 2009). Based on Grueter et al. (2010), increased levels of anandamide after URB597 might activate both CB1 and TRPV1 receptors. Though TRPV1 receptors seem not to affect the rewarding effects of cocaine, they have been shown to reduce cocaine-induced but not cue-induced reinstatement of cocaine self-administration (Adamczyk et al., 2012). We cannot exclude that, in our experimental conditions, anandamide released by cocaine with its levels magnified by URB597, had effects on TRPV1. While we cannot address in this report the involvement of TRPV1 receptors on cocaine-induced sensitization, it is important to note that in the report by Grueter et al. (2010) the effects of anandamide in the NAC were qualitatively similar on both presynaptic-CB1 and postsynaptic-TRPV1 receptors. Further, anandamide has an approximate 10-fold preference for CB1 compared to TRPV1 receptors (Ross et al., 2001; Toth et al., 2009) and the present results show a dose-dependent blockade of cocaine behavioral and neurochemical sensitization with selective occupancy of CB1Rs by rimonabant. Thus, though rimonabant was not tested against URB597, the results strongly suggest an involvement of TRPV1 receptors in cocaine sensitization would not interfere with that presently found for CB1Rs. Thus, we believe that the interaction of cannabinoid CB1 and DA D1/D2 receptor activation and the enhancement of NAC core neurons firing (Gerdeman et al., 2002; Seif et al., 2011) are in perfect agreement with our findings that endocannabinoid release and CB1 activation might be required at the early stages of cocaine-induced sensitization in specific brain regions.

There is active debate about the importance and role of sensitization in the transition from simple drug use to drug abuse and dependence (Di Chiara, 2002; Kauer and Malenka, 2007; Robinson and Berridge, 1993). Cocaine sensitization is paralleled by the development of neuronal adaptations within the NAC and the ventral tegmental areas that might facilitate drug seeking (e.g. Bowers et al., 2010; Li et al., 2004; Robinson and Berridge, 1993, 2003; Vanderschuren and Kalivas, 2000). The differences in core and shell neurochemical sensitization (Cadoni et al., 2000; Pierce et al., 1996, and present results) might underlie important behavioral and neurobiological aspects of the transition from simple drug use to subsequent abuse, drug-seeking, and dependence (Di Chiara, 2002; Di Chiara et al., 1999). The occurrence of cocaine-induced sensitization of DA transmission in the NAC core might be of relevance to the habit-forming effects of drugs (Everitt and Robbins, 2005) which have been suggested to be mediated by extrapyramidal striatal areas more related to core than shell nuclei (Belin and Everitt, 2008; Di Chiara, 2002). These functions may include, for example, acquisition or maintenance of behaviors that allow the organism to come in repeated contact with the drug and associated stimuli (drug-seeking behavior) (Di Chiara, 2002; Di Chiara et al., 1993; Everitt and Robbins, 2005).

Cocaine has been suggested to release endocannabinoids (Centonze et al., 2004; Cheer et al., 2007, but see Caille et al., 2007). Blockade of cannabinoid receptors before the sensitizing injection of cocaine in this report significantly attenuated the occurrence of behavioral/neurochemical sensitization. Thus, our results support the hypothesis that neuroadaptations induced by a single injection of cocaine (Ungless et al., 2001) require the release of endocannabinoids. The present results are in agreement with our working hypothesis that: 1) cocaine induces region-specific on-demand release of endocannabinoids, 2) antagonism of the endocannabinoids significantly reduces neurochemical and consequently behavioral sensitization induced by cocaine, and 3) blockade of endocannabinoid degradation facilitates the sensitizing effects of cocaine administered at sub-optimal doses. Taken together the present results suggest that the endocannabinoid system plays a primary role in mediating the inception of long-term brain-adaptive responses to acute stimulant effects, which in turn might alter neuronal pathways that increase a subject's vulnerability to substance use

disorders (Di Chiara, 2002; Koob and Volkow, 2010).

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Supplementary Material

Supp Fig S1-S2

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Acknowledgments

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All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, and the Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1997. The animal facility was accredited by AAALAC International.

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Footnotes

Authors contribution. VT MM JLK and GT conceived and designed the experiments. VT MM AT JG LC and GT carried out the experiments. GT supervised all the experiments. JLK contributed materials and supervised the behavioral experiments. VT MM AT LC JLK and GT analyzed the data, and VT MM JLK and GT contributed to interpretation of findings and drafted the manuscript. All authors critically reviewed the first draft, edited, gave comments and suggestions that resulted in the final submitted version of the paper.

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CB1 receptor antagonism blocks stress-potentiated reinstatement of cocaine seeking in rats

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Abstract

Rationale

Under some conditions stress, rather than directly triggering cocaine seeking, potentiates reinstatement to other stimuli, including a sub-threshold cocaine dose. The mechanisms responsible for stress-potentiated reinstatement are not well-defined. Endocannabinoid signaling is increased by stress and regulates synaptic transmission in brain regions implicated in motivated behavior.

Objectives

To test the hypothesis that cannabinoid receptor 1 (CB1R) signaling is required for stress-potentiated reinstatement of cocaine seeking in rats.

Methods

Following i.v. cocaine self-administration (2 hr access/day) and extinction in male rats, footshock stress alone does not reinstate cocaine seeking, but reinstatement is observed when footshock is followed by an injection of an otherwise subthreshold dose of cocaine (2.5 mg/kg, i.p.). CB1R involvement was tested by systemic administration of the CB1R antagonist AM251 (0, 1, or 3 mg/kg, i.p.) prior to testing for stress-potentiated reinstatement.

Results

Stress-potentiated reinstatement was blocked by both 1 and 3 mg/kg AM251. By contrast, AM251 only attenuated food-reinforced lever pressing at the higher (i.e., 3 mg/kg) dose and did not affect locomotor activity at either dose tested. Neither high-dose cocaine-primed reinstatement (10 mg/kg, i.p.) nor footshock stress-triggered reinstatement following long-access cocaine self-administration (6 hr access/day) were affected by AM251 pretreatment. Footshock stress increased concentrations of both endocannabinoids, N-arachidonylethanolamine and 2-arachidonoylglycerol, in regions of the prefrontal cortex.

Conclusions

These findings demonstrate that footshock stress increases prefrontal cortical endocannabinoids and stress-potentiated reinstatement is CB1R-dependent, suggesting that CB1R is a potential therapeutic target for relapse prevention, particularly in individuals whose cocaine use is stress-related.

Keywords: addiction, cocaine, stress, reinstatement, relapse, endocannabinoid, self-administration

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INTRODUCTION

The sudden and often unpredictable relapse to drug use that can emerge even after prolonged periods of drug abstinence is a major obstacle to the effective long-term management of substance abuse. The ability to prevent relapse has been hindered by an imprecise and incomplete understanding of situations that lead to the re-emergence of drug-seeking behavior and the underlying neurobiological processes.

Stress plays a particularly problematic role in drug relapse (McReynolds et al. 2014; Shaham et al. 2000) as it is prevalent and unavoidable in daily life. While it is evident that stress contributes to relapse, the manner in which it does so appears to be more complex than simply triggering use. For example, while clinical laboratory studies have found that stress imagery can trigger craving (Sinha 2009), self-reports by addicts regarding the role of stress in their drug use are suggestive of a less direct relationship between stress and relapse (Furnari et al. 2015; Preston and Epstein 2011). Consistent with this more nuanced role for stress, others have reported that stress may interact with other relapse triggers to promote drug use (Coffey et al. 2002; De La Garza et al. 2009; Duncan et al. 2007). These reports parallel findings from preclinical studies showing that acute stressors can potentiate reinstatement induced by cocaine-related cues in rats (Buffalari and See 2009; Feltenstein and See 2006).

Previous studies from our laboratory have demonstrated that the ability of intermittent electric footshock to reinstate extinguished cocaine seeking in rats can vary depending on the prior history of cocaine use. We have identified conditions under which rats with a history of extended access cocaine self-administration (14 × 6 hrs/day) show robust footshock-triggered reinstatement of cocaine seeking following extinction, while rats that have a history of more limited daily access (14 × 2 hrs/day) do not (Mantsch et al. 2008a). However, although footshock stress does not directly reinstate cocaine seeking following self-administration under these short-access conditions, it can amplify the effects of an otherwise sub-threshold priming injection of cocaine (2.5 mg/kg; Graf et al. 2013), thereby potentiating reinstatement. We have proposed that this potentiating effect of footshock stress reflects its role in many drug users in whom stress appears to "set the stage" for cocaine use. In contrast to the relapse-triggering effects of stress, which have been extensively studied, relatively little is known about the neurobiological mechanisms that underlie these stage-setting effects of stress.

We recently reported that stress-potentiated reinstatement, unlike stress-triggered reinstatement (Mantsch et al. 2008a), is corticosterone-dependent, and that elevated corticosterone is sufficient to reproduce the potentiating effect of stress (Graf et al. 2013). Moreover, we found that one corticosterone-dependent mechanism through which stress potentiates cocaine-induced reinstatement involves reduced dopamine clearance in the nucleus accumbens by the glucocorticoid-sensitive organic cation transporter 3 (OCT3; Graf et al. 2013). Endocannabinoid (eCB) signaling represents a second corticosterone-dependent mechanism through which stress could potentiate cocaine-induced reinstatement. Both stress and corticosterone increase eCB levels in brain regions that have been implicated in drug-seeking behavior, most notably the prefrontal cortex (PFC) and nucleus accumbens (NAc; Hill et al. 2011; Rademacher et al. 2008). In the brain, the CB1Rs are thought to represent the primary targets for eCB signaling (Devane et al. 1988; Egertova et al. 1998; Herkenham et al. 1991; Howlett et al. 2002; Pertwee 1997; Pertwee and Ross 2002), although CB2R-mediated effects have been reported (Brusco et al. 2008; Onaivi 2011; Onaivi et al. 2006). As CB1Rs have been proposed to play a critical role in both drug addiction (Sidhpura and Parsons 2011) and adaptation to stress (Hill et al. 2010), we hypothesize that stress potentiation of cocaine-induced reinstatement requires eCB signaling through CB1Rs. In the present study we test this hypothesis by assessing the effects of the CB1R antagonist, AM251, on stress-potentiated reinstatement.

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METHODS

Subjects

Male Sprague-Dawley rats (275–300 g at arrival), obtained from Harlan Laboratories, were individually housed in a humidity- and temperature-controlled AAALAC-accredited facility with food and water available *ad libitum*. Rats were maintained on a 12h/12h reverse light/dark cycle (0700–1900 lights off) and all behavioral procedures were conducted during the dark phase of the light cycle. All experimental procedures were carried out in compliance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee (Marquette University).

Catheter implantation

For intravenous self–administration and reinstatement testing, each rat was anesthetized with ketamine HCl (100 mg/kg, i.p.) and xylazine (2 mg/kg, i.p.) and had an indwelling silicon (Silastic; Dow Corning; 0.64 mm i.d.; 1.19 mm o.d.) catheter implanted into the jugular vein. Catheters were connected to back mount cannulae (Plastics One) attached to monofilament surgical mesh (Atrium Medical). When placed in the operant chambers, the back mount cannulae was connected to PE tubing (0.51 mm i.d. × 1.52 mm o.d.) encased in stainless-steel spring leashes (Plastics One). The PE tubing was connected via a fluid swivel (Instech Lab) suspended above the self–administration chamber and Tygon tubing (VWR; 0.51 mm i.d. × 1.52 mm o.d.) to syringes in pumps (Razel) outside of the operant chamber. Rats received ibuprofen in their drinking water for 3 days following surgery and were given iv antibiotic treatment for at least 5 days. The rats were allowed to recover for 1 week before the initiation of self–administration training.

Cocaine Self-Administration

Self-administration/reinstatement procedures were conducted in computer-interfaced operant conditioning chambers equipped with retractable levers and stimulus lights above each lever in sound-attenuating cubicles (MED-Associates). Following recovery from surgery, rats were trained to self-administer cocaine (0.5 mg/kg/infusion) by pressing a lever under a fixed-ratio (FR) 1 schedule during daily 2-h sessions. Pressing the lever resulted in an infusion (200 μL over 5 s) followed by a 10 s time-out period during which lever presses were recorded but not reinforced and the stimulus light above the lever was extinguished. Responding on a second lever, the inactive lever, was also recorded, but not reinforced. Following stable responding, the FR value was increased gradually to FR4. Once stable responding on FR4 was achieved (<10% change over 3 days), rats were allowed to self-administer cocaine daily for 14 additional days.

Extinction and reinstatement

After 14 days of cocaine self-administration, rats underwent extinction training which was identical to cocaine self-administration with the exception that the cocaine was replaced with saline. Rats underwent daily 2-h extinction training sessions until they met the extinction criterion (<15 lever presses/2 h). Once rats met this criterion, a reinstatement test was given the next day. Aside from footshock and/or cocaine pre-exposure, reinstatement sessions were otherwise identical to extinction conditions. All rats underwent multiple reinstatement tests within each experiment in a counterbalanced fashion. Each rat received a maximum of 4 reinstatement tests. Rats were given additional extinction/washout sessions between reinstatement tests until they reached criterion (<15 lever presses/2 h) before receiving additional reinstatement tests.

Experiment 1: Effect of CB1R antagonism on stress-potentiated reinstatement of cocaine seeking

Under the self–administration parameters used in the present study (0.5 mg/kg/inf; 14 × 2 hrs/day), we have found previously that footshock alone does not trigger reinstatement of extinguished cocaine seeking, but potentiates reinstatement in response to an injection of an otherwise subthreshold dose of cocaine (2.5 mg/kg, i.p.; Graf et al. 2013). Following self-administration and extinction, all rats were tested for reinstatement under each of the following conditions, in counter-balanced sequence: 1) no footshock/cocaine; 2) footshock/saline; and 3) footshock/cocaine following 30-min pretreatment with the CB1R antagonist, AM251 (1 or 3 mg/kg, i.p.), or vehicle (1:1:18 ethanol: Cremaphor: saline). Separate sets of rats were used for each dose of AM251 so each rat received 4 reinstatement tests total. Intermittent electric footshock (3 × 0.5 mA, 200 ms duration, mean intershock interval 40s, range 10–70s over a 15 min period) was delivered through the stainless-steel grid floors of the operant conditioning chambers immediately prior to cocaine (2.5 mg/kg, i.p.) or saline (0.9% NaCl) injections followed by the 2-h reinstatement test sessions.

Experiment 2: Effect of CB1R antagonism on cocaine-primed and stress-triggered reinstatement of cocaine seeking

We have previously reported that stress-potentiated reinstatement is mediated in part by a mechanism that is distinct from that involved in either stress-triggered or cocaine-primed reinstatement; stress-potentiated reinstatement is corticosterone-dependent (Graf et al. 2013), while stress-triggered and cocaine-primed reinstatement are not (Erb et al. 1998; Graf et al. 2011; Mantsch et al., 2008b). To determine if CB1R involvement is also selective for stress-potentiated cocaine seeking, we tested separate groups of rats for the effects of AM251 on cocaine-primed and stress-triggered reinstatement.

To test for the effect of AM251 on cocaine-primed reinstatement, rats underwent cocaine self-administration and extinction as described above, but were given a systemic injection of a dose of cocaine (10 mg/kg, i.p) which has been reported to reliably reinstate cocaine seeking (Mantsch et al. 2008b; Mantsch and Goeders 1999; Mantsch et al. 2004), or saline immediately prior to the start of the 2-h reinstatement session. As above, rats were pretreated with AM251 (1, 3 mg/kg, ip) or vehicle (1:1:18 ethanol: Cremaphor: saline) 30 min prior to the start of the reinstatement test. Each rat was tested for reinstatement under each of the following conditions in counterbalanced condition: 1) vehicle/saline; 2) vehicle/cocaine; 3) AM251/cocaine. Rats were given extinction/washout sessions between reinstatement tests until they reached criterion (<15 lever presses/2 h) before receiving additional reinstatement tests. Each rat received all reinstatement tests in a counterbalanced order and received a total of 3 reinstatement tests.

To determine if CB1R activation represents a distinct mechanism that underlies the ability of stress to potentiate, rather than directly trigger induce reinstatement, we tested a separate group of rats for the effect of AM251 under conditions in which footshock stress alone can trigger reinstatement of extinguished cocaine seeking. We have previously shown that delivery of footshock alone produces reliable and robust reinstatement of cocaine seeking in rats with a history of longer daily access to cocaine self-administration (6 hrs/day; Mantsch et al. 2008a; Blacktop et al. 2011; Vranjkovic et al. 2014). In these experiments, rats were provided with daily long access (6 hr/day × 14 days) to cocaine (0.5 mg/kg/inf) for self-administration prior to extinction (as described above) and testing for footshock stress-triggered reinstatement (same footshock conditions as above). To test for CB1R involvement in stress-triggered reinstatement, rats were pretreated with either AM251 (1 mg/kg, ip) or vehicle (1:1:18 ethanol: Cremaphor: saline) 30 min prior to delivery of footshock followed by reinstatement testing. Each rat was tested under the following reinstatement conditions, in a counterbalanced order: 1) vehicle/no footshock; 2) vehicle/footshock; 3) AM251/no footshock; 4) AM251/footshock. Rats were given extinction/washout sessions between reinstatement tests until they reached criterion (<15 lever presses/2 h) before receiving additional reinstatement tests. Each rat received a total of 4 reinstatement tests and was required to once again meet the extinction criterion (<15 lever presses/2h) between tests.

Experiment 3: Effect of CB1R antagonism on responding for food and basal locomotor activity

Although the lack of AM251 effects on cocaine-primed and stress-triggered reinstatement suggests that the effects of the drug on stress-potentiated reinstatement were not secondary to non-specific motor suppression, we decided to further address this possibility by testing a separate group of rats for AM251 effects on food-reinforced responding and locomotor activity. When testing for effects on food-reinforced behavior, rats were food deprived to 90% of their starting body weight and trained to press a lever under a FR4 schedule of sucrose-sweetened food pellet (45 mg; BioServ)

reinforcement during daily 30-min sessions. Drug effects were tested once stable responding was achieved (<10% change over 3 days). AM251 (1, 3 mg/kg, ip) or vehicle (1:1:18 ethanol: Cremaphor: saline) was administered 30 min prior to the start of the session. Each rat received vehicle and both doses of AM251 in a counterbalanced order for a total of 3 tests. Rats were given additional days between drug tests to ensure that stable food responding was once again achieved before the next test.

As there is strong evidence for a role of endocannabinoids in feeding, metabolism, and obesity (Bellocchio et al. 2006; Kirkham and Tucci 2006; Silvestri and Di Marzo 2013), food responding alone may not be the most accurate measurement of CB1R antagonist effects on motor activity. Therefore, the effect of AM251 on basal locomotor behavior was also determined. Locomotor activity was measured in the home cages of the rats using an automated activity system (AccuScan Instruments, Inc., Columbus, OH) consisting of photocell-containing frames, equipped with sensor panels containing 16 photo beams in a horizontal orientation, into which cages could be placed for recording. Both total (total photobeam breaks) and ambulatory (consecutive photobeam) breaks activity counts were recorded using a computer interfaced MicroMax software-based system (AccuScan Instruments). Prior to testing for drug effects, rats received 3 days of systemic i.p. saline injections to habituate them to injections and limit the contribution of novelty/injection stress-related effects. Thereafter, all rats were tested for effects of AM251 (1 and 3 mg/kg, ip) and vehicle (1:1:18 ethanol: Cremaphor: saline) in a counterbalanced sequence with washout days between each test. Locomotor activity was recorded for one hour starting immediately post-injection.

Experiment 4: Effect of footshock stress on endocannabinoid content in the medial prefrontal cortex

The medial prefrontal cortex (mPFC) is critical for cocaine seeking in response to multiple reinstating stimuli, including footshock and cocaine (McFarland et al. 2004). Subregions of the mPFC (e.g., prelimbic, infralimbic cortices, anterior cingulate) appear to have differential roles in reinstatement following exposure to stress (Capriles et al. 2003) or cocaine (Capriles et al. 2003; Peters et al. 2008). While it has been reported that stress can increase endocannabinoid content in the mPFC (Hill et al. 2011), most studies examining the effect of stress on eCB content have utilized acute restraint or forced swim as stressors (Hill et al. 2011; McLaughlin et al. 2012; Rademacher et al. 2008; Roberts et al. 2012). We conducted a preliminary analysis of the effects of footshock stress on eCB content in subregions of the mPFC in rats with no history of cocaine self-administration. Rats were handled for 2 min/day for 3 days prior to exposure to the footshock. On the day of testing, rats were transported to the experimental room 1-h prior to administration of footshock stress. Rats were placed into the operant conditioning chambers and received the same 15-min footshock stress that was administered during reinstatement testing: control rats were placed in the operant chambers for 15 min but did not receive the stressor. Immediately after the 15-min footshock session, rats were placed back into their home cages and 15 min later were rapidly decapitated under isoflurane anesthesia (30-s exposure) for tissue collection. Brains were rapidly removed (2.5 min total time) and flash frozen by submersion in liquid nitrogen for 25s and then stored at −80°C.

For tissue dissection, 1 mm prefrontal cortical sections (approx. 3.72 mm to −2.76 mm from Bregma) were isolated from coronal sections on dry ice. Prefrontal cortical subregions (prelimbic (PL), infralimbic (IL), and anterior cingulate cortices (AC)) were dissected bilaterally using a scalpel blade, weighed and collected in a microcentrifuge tube and stored at −80°C. Subregions from two animals were pooled for each data point.

Endocannabinoid Liquid Chromatography/Mass Spectrometry

Concentrations of the endocannabinoids N-arachidonylethanolamine (AEA) and 2-arachidonylglycerol (2-AG) were extracted from tissue using a protocol adapted from Patel et al. 2003. Briefly, tissue samples were homogenized in 2mL of acetonitrile with 84 pmol [$^2H_8$]AEA and 186pmol [$^2H_8$]2-AG using glass rods. Samples were sonicated for 60 min and frozen overnight at −20°C to precipitate proteins. The supernatant was obtained following centrifugation at 1500 × *g* for 2 min at 4°C, was dried under N_2 and the lipids were resuspended in methanol. The two endocannabinoids were isolated and quantified by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS; Agilent LC-MSD 1100 series) and quantified by isotope dilution as described previously (Patel et al. 2003).

Drugs

Cocaine HCl was obtained from the National Institute on Drug Abuse (NIDA) through the NIDA Drug Supply Program. The CB1R antagonist AM251 was purchased from Sigma-Aldrich. Cocaine was dissolved in saline (0.9% bacteriostatic saline). AM251 was dissolved first in ethanol, followed by Cremaphor and finally by saline (0.9% bacteriostatic saline) in a 1:1:18 ratio.

Statistical Analysis

Statistical analyses were conducted using SPSS statistics software and consisted of one- or two-way repeated measures ANOVA followed, when appropriate, with post-hoc testing using Bonferroni-corrected t-tests. For all analyses, statistical significance was defined as $p<0.05$.

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RESULTS

The numbers of cocaine infusions and responses during the self-administration and extinction sessions for each reinstatement experiment are shown in Table 1. Rats showed consistent responding during self-administration between self–administration day 1 and 14 in experiments 1 and 2. Following completion of cocaine self-administration, rats showed a reduction in lever presses across extinction sessions in both experiments and took an average of 7.67 ± 1.38 extinction sessions before reaching criteria.

Table 1

Cocaine self-administration and extinction responding

	Cocaine SA		Extinction	
SA Day 1	**SA Day 14**	**First Ext**	**Last Ext**	
1 ShA; (12)	121.4 ± 7.5	126.8 ± 7.8	75.9 ± 7.2	9.9 ± 1.0
2 ShA; (8)	117.3 ± 10.6	120.0 ± 11.0	77.4 ± 19.3	9.1 ± 1.8
LgA; (5)	317.8 ± 34.2	396.0 ± 12.54	166.8 ± 41.2	7.6 ± 2.6

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Data represent total active lever responses/2-h session ± SEM for experiments 1 and 2 for short access (ShA) experiments, and lever responses/6-hr session ± SEM for experiment 2 for long-access (LgA) experiments during days 1 and 14 of cocaine self-administration (SA) and total active lever responses/2-h session ± SEM on the first day of extinction (Ext) and the last day of extinction before reinstatement testing for experiments 1 and 2.

Experiment 1: Effect of CB1R antagonism on stress-potentiated reinstatement of cocaine seeking

To confirm that stress produced a potentiation of cocaine-induced reinstatement, as previously described (Graf et al. 2013) we initially compared reinstatement following administration of the footshock/cocaine combination with reinstatement following footshock/saline or administration of cocaine alone (no footshock/cocaine) only in rats pretreated with the vehicle for AM251 (1:1:18 ethanol: Cremaphor: saline). As previously reported

in rats tested under the same self-administration conditions (Graf et al. 2013), neither low-dose cocaine (2.5 mg/kg, i.p.) nor footshock alone reinstated cocaine seeking. However, robust reinstatement was observed when the two stimuli were combined (Fig. 1). A two-way repeated measures ANOVA showed significant main effects of reinstatement testing (extinction vs. reinstatement; $F(1,22)=45.26$, $p<0.001$) and reinstatement condition (footshock/saline, no footshock/cocaine, footshock/cocaine; $F(2,22)=24.42$, $p<0.001$) and a significant interaction between the reinstatement day and reinstatement condition ($F(2,22)=25.52$, $p<0.001$). Post-hoc testing revealed significant reinstatement (compared to extinction responses) following delivery of the footshock/cocaine combination ($p<0.001$) but not with footshock/saline or cocaine alone. Moreover, while no differences in extinction responding were observed across treatment conditions, responding during the reinstatement session that followed delivery of the footshock/cocaine was increased compared to responding following footshock/saline or cocaine alone ($p<0.001$).

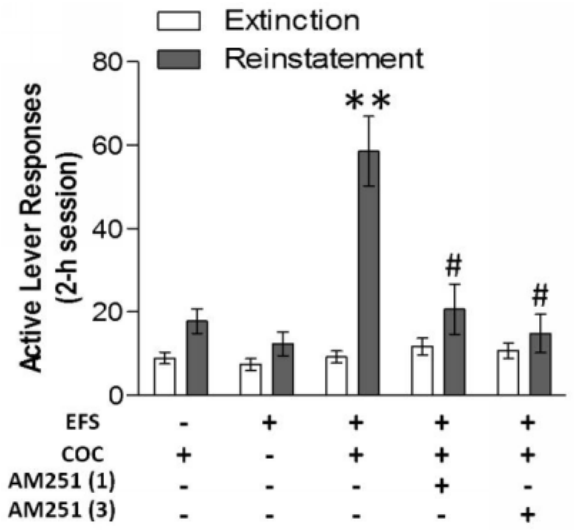

Figure 1

Stress-potentiated reinstatement is CB1R-dependent. Data represent mean active lever responses (± SEM) during 2-h extinction and reinstatement tests. Stress-potentiated reinstatement is observed when rats are exposed to a stressor, 15 min intermittent electric footshock stress (EFS) followed immediately by a subthreshold dose of cocaine (COC, 2.5 mg/kg, i.p.; **p<.01, compared to reinstatement). Stress-potentiated reinstatement is blocked by pre-treatment with the CB1R antagonist AM251 (1 (n=6), 3 (n=6) mg/kg, i.p.) 30 min prior to the reinstatement test (#p<.05 compared to Veh/EFS/COC).

The effects of 1 mg/kg AM251 and vehicle (Veh) and 3 mg/kg AM251 and vehicle were assessed in different groups of rats, so a separate 2-way repeated measures (footshock/cocaine x AM251 pretreatment) ANOVA was conducted for each dose. Both doses of AM251 prevented stress-potentiated reinstatement. For 1 mg/kg AM251 doses, 2-way repeated measures ANOVA showed an overall significant effect of reinstatement (Extinction vs. Reinstatement; 1 mg/kg: $F(1,5)=26.86$, $p<0.01$), but not drug (Veh vs. AM251; $F(1,5)=5.03$, $p=0.08$) and a significant interaction between the reinstatement day and drug ($F(1,5)=9.74$, $p<0.05$). Post-hoc testing showed that reinstatement was observed in Veh-pretreated ($p<0.05$), but not AM251 pretreated rats and that reinstatement following vehicle pretreatment was significantly increased relative to reinstatement following AM251 ($p<0.05$). Similarly, for the 3 mg/kg AM251 dose, 2-way repeated measures ANOVA revealed significant overall effects of reinstatement testing ($F(1,5)=14.02$, $p<0.05$) and AM251 pretreatment ($F(1,5)=12.18$, $p<0.05$) and a significant reinstatement x pretreatment interaction ($F(1,5)=18.24$, $p<0.01$). As with the lower dose, post-hoc testing demonstrated that reinstatement was observed in Veh-pretreated rats ($p<0.05$), but not rats pretreated with 3 mg/kg AM251 and that reinstatement following vehicle pretreatment was significantly increased relative to reinstatement following 3 mg/kg AM251 ($p<0.05$).

Experiment 2: Effect of CB1R antagonism on cocaine-primed and stress-triggered reinstatement of cocaine seeking

In contrast to stress-potentiated cocaine-induced reinstatement, AM251 did not prevent reinstatement in response to a 10 mg/kg i.p. priming dose of cocaine (Fig. 2A). Effects of pretreatment with 1 mg/kg and 3 mg/kg AM251 on cocaine-primed reinstatement were tested in separate groups of rats. In both cases 2-way repeated measures reinstatement (reinstatement vs. extinction) x drug pretreatment (AM251 vs. Veh) ANOVA revealed overall reinstatement effects ($F_{(1,7)}=18.62$, $p<0.001$ and $F_{(1,7)}=25.51$, $p<0.01$) but not overall effects of AM251 and no reinstatement x AM251 pretreatment interactions.

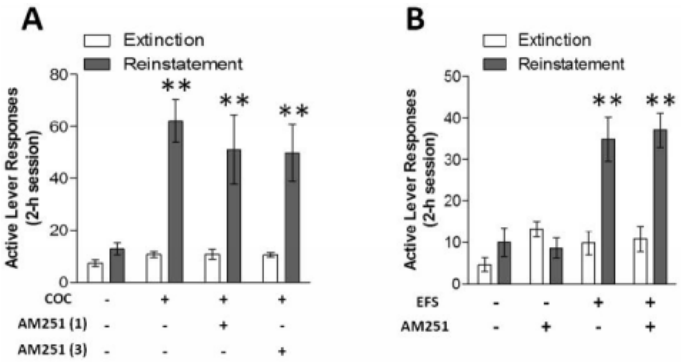

Figure 2

Cocaine-primed or stress-induced reinstatement is not CB1R-dependent. Data represent mean active lever responses (± SEM) during 2-h extinction and reinstatement tests. A. Cocaine-primed reinstatement is observed when rats are given a systemic injection of a high dose of cocaine (COC, 10 mg/kg, i.p.; **p<.01 compared to extinction). Cocaine-primed reinstatement is not significantly attenuated by pre-treatment with the CB1R antagonist, AM251 (1 (n=8), 3 (n=8) mg/kg, i.p.) 30 min prior to the cocaine injection. B. Stress-induced reinstatement is observed when rats are given electric footshock stress (EFS) for 15 min immediately followed by the reinstatement session in rats with a history of long-access self-administration (**p<.01 compared to extinction). Stress-induced reinstatement is not significantly attenuated by pretreatment with AM251 (1 mg/kg, i.p.; n=5) 30 min prior to EFS.

While stress-potentiated reinstatement in short-access rats was blocked by pretreatment with AM251 (1 mg/kg, ip), stress-triggered reinstatement in

long-access rats was unaffected by the same treatment (Fig 2B). A 2-way repeated measures drug pretreatment (Veh vs AM251) x reinstatement (no footshock vs footshock) ANOVA revealed overall reinstatement effects ($F_{(1,4)}$=37.16, p<0.01) but no overall effects of AM251 and no reinstatement x AM251 pretreatment interactions. Post-hoc testing demonstrated that reinstatement was observed in both Veh- (p <0.01), and AM251-pretreated rats (p<0.01) but that reinstatement following vehicle pretreatment was not significantly different from reinstatement following 1 mg/kg AM251 (p>0.05).

Experiment 3: Effect of CB1R antagonism on responding for food and basal locomotor activity

AM251 at a dose of 3 mg/kg produced dose-dependent reductions in food-reinforced lever pressing while 1 mg/kg AM251 was without significant effect (Fig. 3). A one-way repeated measures ANOVA revealed a significant AM251 effect on food-reinforced responding (Veh, 1 mg/kg AM251, 3 mg/kg AM251; $F_{(2,12)}$=4.41, p<0.05) and post hoc testing demonstrated that pretreatment with 3 mg/kg, but not 1 mg/kg AM251 significantly attenuated food-reinforced lever responding compared to vehicle pretreatment (p<0.05).

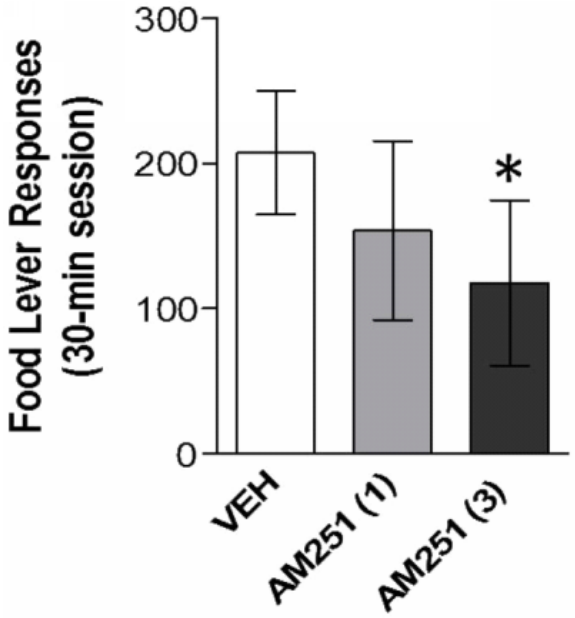

Figure 3

Food-reinforced lever pressing is attenuated by pre-treatment with the CB1R antagonist AM251. Data are presented as mean food lever responses during the 30-min session ± SEM. Pre-treatment with AM251 30 min prior to the beginning of the session significantly attenuated food-reinforced lever pressing at the higher dose (3 mg/kg, i.p), but not the low dose (1 mg/kg, i.p.; n=7; *p<.05 compared to Veh). Each rat received each drug treatment and the order of drug treatment was counterbalanced.

AM251 effects on basal locomotor activity were tested (Table 2). A one-way repeated measures ANOVA did not reveal a significant effect of the drug (Veh, 1 mg/kg AM251, 3 mg/kg AM251) for either total activity ($F_{(2,10)}$=2.56, p=0.13) or ambulatory movement ($F_{(2,10)}$=0.81, p=0.47) consistent with previous findings (Adamczyk et al. 2012).

Table 2

Effects of CB1R antagonism on locomotor activity

	1st 30 min		2nd 30 min		Total 1 hr	
	Total	**Ambulatory**	**Total**	**Ambulatory**	**Total**	**Ambulatory**
Vehicle	1632 ± 286	701 ± 122	611 ± 138	301 ± 64	2244 ± 249	1003 ± 122
AM251 (1)	1539 ± 280	681 ± 104	854 ± 231	323 ± 75	2393 ± 435	1005 ± 149
AM251 (3)	2058 ± 283	776 ± 114	1391 ± 257	484 ± 70	3450 ± 477	1261 ± 163

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Data represented are mean activity counts ± SEM for total (total beam breaks) and ambulatory (consecutive beam breaks) activity. The data are presented as activity during the 1st 30 min, 2nd 30 min, and total 1-h after the injection of either Vehicle (1:1:18 ethanol: Cremaphor: saline) or AM251 (1, 3 mg/kg, i.p.). Systemic administration of AM251 did not significantly affect total or ambulatory activity (n=6) during the 1-h after the injection.

Experiment 4: Effect of footshock stress on endocannabinoid content in the medial prefrontal cortex

The effects of footshock stress on contents of AEA and 2-AG in subregions of the medial prefrontal cortex were examined in drug-naïve rats euthanized 15 min after the footshock period ended (Table 3). A 2-way ANOVA with footshock and brain region as factors revealed an overall significant effect of footshock on AEA content ($F_{(1,36)}$=8.411 p<.01) and an overall effect of brain region ($F_{(1,36)}$ = 18.10, p<.001) on 2-AG content. We also ran planned comparisons in each region for the effect of stress on AEA or 2-AG in each region. Despite trends in each region AEA was not significantly increased in any brain region (PL: p=0.10; IL: p=0.12; AC: p=0.20) but 2-AG content was significantly increased in the AC as a result of stress (p<.05).

Table 3

Effects of footshock stress on endocannabinoid content in subregions of the medial prefrontal cortex

	2-AG (nmol/g tissue)		AEA (pmol/g tissue)	
	Control	**Shock**	**Control**	**Shock**
Anterior Cingulate	2.4 ± 0.6	5.6 ± 0.5	8.9 ± 0.9	19.3 ± 5.1

Prelimbic	11.0 ± 1.8	9.6 ± 1.5	7.1 ± 2.7	17.8 ± 5.4
Infralimbic	4.6 ± 0.7	5.5 ± 0.8	6.3 ± 1.0	16.8 ± 7.1

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Data represented are mean concentrations of the endocannabinoids 2-arachidonylglycerol (2-AG; nmol/g tissue) and anandamide (AEA; pmol/g tissue) 15 min after termination of a 15 min-footshock (n=7) or in control rats (n=7). There was a significant effect of footshock stress on AEA levels across the 3 brain regions ($p<.001$). Footshock stress significantly increased 2-AG in the anterior cingulate cortex ($p<.05$) and levels of 2-AG in the prelimbic cortex are significantly higher than those in the anterior cingulate or infralimbic ($p<.001$).

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DISCUSSION

Under conditions where it does not directly trigger relapse, stress can promote responsiveness to other stimuli, thereby "setting the stage" for drug use. We have previously modeled this stage-setting effect using a self-administration/approach in which footshock stress augments cocaine-induced reinstatement in rats (Graf et al. 2013). We reproduced these findings in the present study, demonstrating that, under the self–administration parameters used (0.5 mg/kg/inf; 14 × 2 hrs/day), footshock alone does not reinstate cocaine seeking, but potentiates reinstatement in response to an injection of an otherwise subthreshold dose of cocaine (2.5 mg/kg, i.p.). We demonstrate that this stage-setting effect of stress is dependent on CB1R signaling. In our paradigm, systemic administration of the CB1R antagonist, AM251, prevents stress-potentiated reinstatement but does not affect reinstatement in response to a priming dose of cocaine (10 mg/kg, ip) or alter reinstatement triggered by shock alone in rats with a history of cocaine self-administration under long-access (daily 6-h/day) conditions. Moreover, although AM251 also attenuated food-reinforced lever pressing, reductions in reinstatement were observed at an AM251 dose that did not alter food-reinforced responding, while reductions in locomotor activity were not observed at any dose tested.

Endocannabinoid signaling in the brain is stress-responsive and is necessary for stress adaptation and coping (Hill et al. 2011; McLaughlin et al. 2012; McLaughlin et al. 2013; McLaughlin et al. 2014). There are two subtypes of cannabinoid receptors: CB1 and CB2. The CB1 receptor is considered to be the predominant cannabinoid receptor in the brain and is expressed in regions critical for motivated behavior, including the prefrontal cortex, nucleus accumbens, and the ventral tegmental area (Devane et al. 1988; Egertova et al. 1998; Herkenham et al. 1991; Melis et al. 2004; Pettit et al. 1998; Wenger et al. 2003), thus positioning CB1Rs as regulators of behavioral processes that enable organisms to effectively respond to stressors and as potential mediators through which stress can influence drug-seeking behavior. Our finding that AM251 prevented the potentiating effects of stress on cocaine-induced reinstatement is consistent with this role for CB1Rs. Moreover, the finding that AM251 did not alter either cocaine-primed or stress-triggered reinstatement in this model suggests that while CB1Rs are important for the interaction between stress and cocaine, they may be less important for the response to either stimuli by itself.

Our study is not the first to examine the contribution of CB1Rs to the reinstatement of extinguished cocaine seeking. De Vries et al. (2001) reported that the CB1R agonist, HU210, reinstated cocaine seeking, while the CB1R antagonist, rimonabant, attenuated cocaine-primed and cue-induced reinstatement but not stress-triggered reinstatement in male Wistar and Long Evans rats. These effects on cocaine-primed reinstatement were reproduced by Xi et al. (2006) and Adamcyzk et al. (2012) using AM251. It is possible that experimental differences, such as rat strains employed (our study is the only one using Sprague Dawley rats), route of administration for the cocaine-prime injection, self-administration parameters, or CB1R antagonist pretreatment times and doses underlie the different responses obtained. However, we are not the first to report as others have also reported a lack of effect of CB1R antagonism on cocaine-primed reinstatement. Another study did report attenuation of cocaine-primed reinstatement by AM251 (Filip et al. 2006), but the AM251 dose at which effects were observed in that study (10 mg/kg, ip) was very high and effects within the AM251 dose range used in the present study were not found. Moreover, Kupferschmidt et al. (2012) found that icv administration of AM251 blocked CRF-induced reinstatement but failed to block footshock- or cocaine-primed reinstatement. In addition, studies using mice have found no effect of CB1R antagonism on cocaine-primed reinstatement following cocaine self-administration (Ward et al. 2009) or cocaine-conditioned place preference (Vaughn et al. 2011). Clarification of the precise conditions under which CB1Rs do and do not contribute to cocaine-primed reinstatement will require further investigation. Notably, CB1R antagonists or negative allosteric modulators have also been reported to inhibit on drug-primed heroin (De Vries et al. 2003; Fattore et al. 2005) and methamphetamine (Jing et al. 2014) reinstatement as well cue-induced reinstatement following alcohol (de Bruin et al. 2011), heroin (De Vries et al. 2003), methamphetamine (Jing et al. 2014) and nicotine (De Vries et al. 2005) self-administration. In contrast to our findings, these results suggest that the involvement of CB1Rs in drug seeking may extend beyond the potentiating effects of stress.

Our finding that AM251 fails to block stress-triggered reinstatement is consistent with studies by De Vries et al. (2001) and Kupferschmidt et al. (2012). We previously reported that the mechanisms responsible for stress-*triggered* reinstatement and those that mediate stress-*potentiated* reinstatement are distinct. For example, eliminating the corticosterone response to stress has no effect on stress-triggered reinstatement of extinguished cocaine seeking (Erb et al. 1998; Graf et al. 2011), but prevents the ability of stress to potentiate low-dose cocaine-induced reinstatement (Graf et al. 2013). Similar to corticosterone, we observe that the CB1R antagonist AM251 blocked stress-potentiated reinstatement, but not cocaine-primed or stress-triggered reinstatement, suggesting that endocannabinoid involvement in cocaine-seeking behavior in these conditions is also more important for the interaction between stress and cocaine than either stimuli alone. This parallel between corticosterone and endocannabinoids is interesting, considering that corticosterone has been reported to regulate endocannabinoid contents in brain regions that have been implicated in cocaine-seeking behavior (Hill et al. 2011; Kalivas and McFarland 2003; McFarland et al. 2004; Rademacher et al. 2008). For example, restraint stress produces glucocorticoid-dependent increases in the endocannabinoid, 2-AG, in the prelimbic cortex, resulting in a CB1R-dependent attenuation of GABAergic neurotransmission that disinhibits pyramidal neuron outputs (Hill et al. 2011) that likely include a key glutamatergic pathway to the nucleus accumbens core that has been implicated in cocaine-seeking behavior (McFarland et al. 2004).

In the present study, we observed that footshock stress increased AEA contents in PL, IL and AC subregions of the medial prefrontal cortex and increased 2-AG content in the AC. These results are consistent with prior studies indicating that the prefrontal cortical endocannabinoid system is modulated by stress and anxiety conditions (Hill et al. 2011; McLaughlin et al. 2012; Morena et al. 2014; Rademacher et al. 2008; Roberts et al. 2012). However, prefrontal cortical responses vary in response to different stressors, whereby AEA is decreased following acute restraint (Hill et al. 2011) and swim stress (McLaughlin et al. 2012), or chronic restraint stress (Rademacher et al. 2008), but is increased following a single footshock (Morena et al. 2014), and 2-AG is increased after acute (Hill et al. 2011) or chronic restraint stress (Rademacher et al. 2008). While most stressors result in decreased AEA, footshock stress appears unique in eliciting increases in AEA in periaqueductal grey (Hohmann et al. 2005), basolateral amygdala, and dorsal hippocampus (Morena et al. 2014). While it is not presently clear why footshock stress produces unique AEA responses, it is possible that that this increase is observed in part as a consequence of pain mechanisms. These data support a role for footshock stress-induced increases in endocannabinoid content in relevant brain regions though these levels were not examined in rats with a history of cocaine self-administration or under reinstating conditions.

Several pieces of evidence indicate that the effects of AM251 to reduce stress-potentiated reinstatement are not the result of non-specific motor effects. However to fully examine that possibility, we initially assessed the effects of AM251 on food-reinforced lever pressing. AM251 dose-dependently suppressed food-reinforced responding. However, while the higher AM251 dose tested (3 mg/kg) reduced both reinstatement and food-reinforced responding, the lower AM251 dose (1 mg/kg) selectively attenuated stress-potentiated reinstatement. These findings are consistent with prior reports showing significantly lower food-reinforced responding when pre-treated with 3 mg/kg but not 1 mg/kg (Adamczyk et al. 2012). Notably, there is strong evidence for endocannabinoid involvement in feeding, metabolism and obesity (Bellocchio et al. 2006; Kirkham and Tucci 2006; Silvestri and Di Marzo 2013), suggesting that the attenuation of food-reinforced responding represents an effect on appetite, and not a motor

effect. Consistent with this possibility, AM251 did not attenuate cocaine-primed or shock-triggered reinstatement. In order to further assess any potential non-specific motor effects of AM251, we also tested rats for effects of AM251 on locomotor activity. Pre-treatment with AM251 had no significant effect on locomotor activity at either dose, which is also consistent with earlier reports (Adamczyk et al. 2012), suggesting that AM251 effects on reinstatement behavior were not the result of non-specific motor suppression. In fact, there was a trend towards increased locomotor activity at the higher AM251 dose. Taken altogether, our findings suggest that endocannabinoid involvement in stress-potentiated reinstatement is specific to the ability of stress to potentiate the responsiveness to subthreshold reinstating stimuli.

The present findings add to a large body of research implicating endocannabinoid signaling in cocaine addiction (see Wiskerke et al. 2008 for review). Notably, polymorphisms of the gene for the CB1 receptor, *CNR1*, are associated with cocaine dependence (Clarke et al. 2013; Lopez-Moreno et al. 2012; Zuo et al. 2007; Zuo et al. 2009) and it has been suggested that circulating endocannabinoids may be a viable biomarker for cocaine use disorder in humans (Pavon et al. 2013). Demonstration of a contribution of endocannabinoid signaling to cocaine use in the context of stress may shed additional light on the complex relationship between endocannabinoids and cocaine addiction. While issues related to psychiatric side effects may limit the clinical utility of CB1R antagonists (Christensen et al. 2007), at minimum these data provide mechanistic insight into the processes through which stress influences drug relapse and therefore may guide future treatment approaches.

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Footnotes

The authors declare no conflicts of interest.

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Cocaine-Induced Reinstatement of Cocaine Seeking Provokes Changes in the Endocannabinoid and *N*-Acylethanolamine Levels in Rat Brain Structures

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Abstract

There is strong support for the role of the endocannabinoid system and the noncannabinoid lipid signaling molecules, *N*-acylethanolamines (NAEs), in cocaine reward and withdrawal. In the latest study, we investigated the changes in the levels of the above molecules and expression of cannabinoid receptors (CB1 and CB2) in several brain regions during cocaine-induced reinstatement in rats. By using intravenous cocaine self-administration and extinction procedures linked with yoked triad controls, we found that a priming dose of cocaine (10 mg/kg, i.p.) evoked an increase of the anadamide (AEA) level in the hippocampus and prefrontal cortex only in animals that had previously self-administered cocaine. In the same animals, the level of 2-arachidonoylglycerol (2-AG) increased in the hippocampus and nucleus accumbens. Moreover, the drug-induced relapse resulted in a potent increase in NAEs levels in the cortical areas and striatum and, at the same time, a decrease in the tissue levels of oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) was noted in the nucleus accumbens, cerebellum, and/or hippocampus. At the level of cannabinoid receptors, a priming dose of cocaine evoked either upregulation of the CB1 and CB2 receptors in the prefrontal cortex and lateral septal nuclei or downregulation of the CB1 receptors in the ventral tegmental area. In the medial globus pallidus we observed the upregulation of the CB2 receptor only after yoked chronic cocaine treatment. Our findings support that in the rat brain, the endocannabinoid system and NAEs are involved in cocaine induced-reinstatement where these molecules changed in a region-specific manner and may represent brain molecular signatures for the development of new treatments for cocaine addiction.

Keywords: cocaine reinstatement, cannabinoid receptors, endocannabinoids, immunohistochemistry, LC-MS/MS method, *N*-acylethanolamines, relapse

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1. Introduction

Cocaine use disorder continues to remain a major world health issue. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) cocaine is the most commonly used illegal stimulant used in Europe [1] and a relapse in taking it belongs to the more difficult challenges undertaken during the treatment for both the patient and the physician. Moreover, the pathophysiology of this brain disorder still remains unclear.

In recent years, the changes in the lipid signaling molecules such as endocannabinoids and *N*-acetylethyloamines (NAEs) in cocaine reward and the drug's withdrawal have been demonstrated [2,3,4,5,6]. In fact, self-administration of cocaine induced both a potent decrease of the anandamide (AEA) levels in the cerebellum and in the 2-arachidonoylglycerol (2-AG) levels in the hippocampus and striatum with fewer cannabinoid CB1 receptors, while increased 2-AG levels were observed in the frontal cortex and cerebellum and were was linked with cocaine reward in rats [2,7]. A cocaine-free period with extinction training resulted in a potent reduction of the endocannabinoid levels in the limbic and subcortical areas with the cannabinoid CB2 receptor downregulation [7].

Apart from endocannabinoids, which are direct agonists of CB receptors, there are also other lipid-based mediators—NAEs (oleoylethanolamide (OEA) and palmitoylethanolamide (PEA))—and they act indirectly on the cannabinoid receptors. NAEs are the endogenous ligands of the nuclear receptor peroxisome proliferator-activated receptors (PPAR-α) and the transient receptor potential cation channel subfamily V member 1 (TRPV1) [8]. PEA and OEA levels could strengthen the effect of AEA on the cannabinoid receptor or TRPV1 ("entourage effect") [9] and consequently potentiate the effect of endocannabinoids [10]. Contingent cocaine administration upregulated the NAEs (oleoylethanolamide (OEA) and palmitoylethanolamide (PEA)) levels in the limbic areas [2], while the levels of these molecules were also seen to increase during extinction in the prefrontal cortex and the hippocampus only during the rats' self-administered cocaine phase [2,7].

In behavioral preclinical studies, the evidence for engagement of the endocannabinoid system on drug-seeking behavior was demonstrated using specific CB1 receptor antagonists that attenuated cocaine-primed and cue-induced reinstatement of cocaine-seeking behavior in rats [11,12,13].

To further examine the involvement of endocannabinoids and NAEs in cocaine addiction, this study attempted to reveal the role of AEA, 2-AG, OEA, and PEA in cocaine relapse. Through using the drug-primed procedure with a yoked triad control with rats, we studied the molecule tissue levels with using liquid chromatography with a tandem mass spectrometry (LC-MS/MS) method. Finally, we examined the cannabinoid CB1 and CB2 receptor brain expression following cocaine reinstatement. We selected several rat brain structures related to substance use disorders including prefrontal cortex (executive control), amygdala and hippocampus (memory and learning), dorsal striatum (habit forming learning), nucleus accumbens (reward processing), ventral tegmental area (reward-related and goal-directed behaviors), and cerebellum (nonmotor function, intertwined with brain reward, motivational drive, saliency, inhibitory control, and insight processes) [14,15,16].

The present study on the role of the endocannabinoid system in the cocaine dependence mechanism may contribute to the many missing questions about cocaine's interaction of the endocannabinoid system, and may help to determine whether the endocannabinoid system is the target point for new addiction treatment drugs in the future.

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2. Results

2.1. Behavioral Studies

After 14 self-administration sessions, the animals showed stable lever-pressing rates during the last 3 self-administration days with less than a 10% difference in their daily intake of cocaine (Figure 1). The mean number of cocaine infusions per day during the last 3 self-administration days varied from 26 to 29 while during 14 experimental sessions the animals received 164.9 ± 9.2 mg/rat of cocaine.

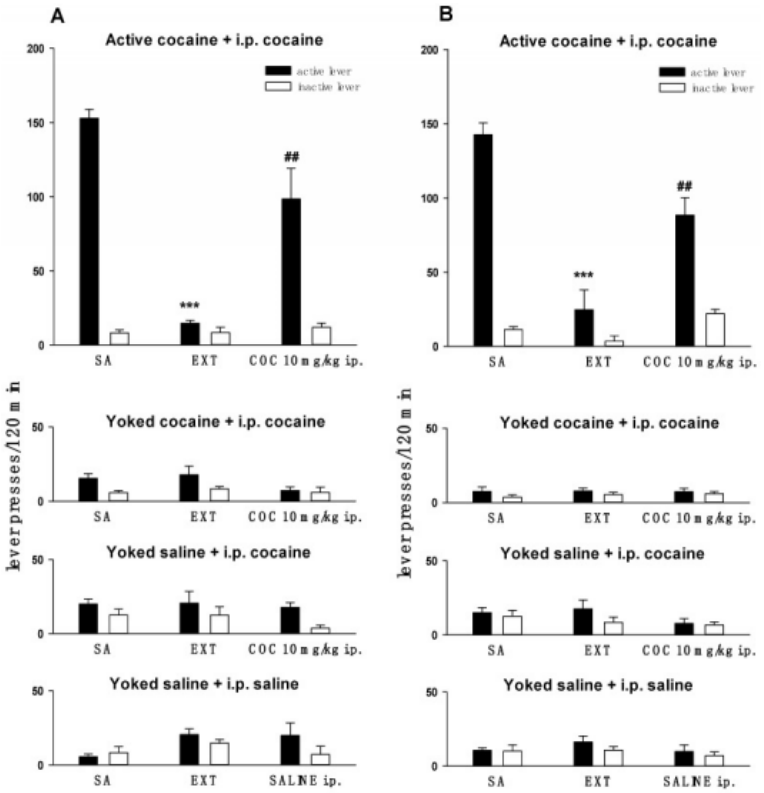

Figure 1

The number (mean ± SEM) of active (black bars) and inactive (white bars) lever presses during the last three self-administration (SA) and extinction (EXT) sessions in rats trained to self-administered cocaine (0.5 mg/kg/infusion) and forced to cocaine priming (COC 10 mg/kg i.p.) during the reinstatement test in active cocaine + i.p. cocaine, yoked cocaine + i.p. cocaine, yoked saline + i.p. cocaine and yoked saline + i.p. saline was shown. Panel **A**—experiment to evaluate the endocannabinoid and NAEs tissue level; panel **B**—experiment to evaluate the expression of cannabinoid receptors. N = 7–8 rats/group. *** $p < 0.001$ vs. active lever SA; $^{\#\#}$ $p < 0.01$ vs. active lever EXT, (Newman–Kelus test).

Figure 1 shows the behavioral responses of the rats that underwent cocaine self-administration, extinction training, and cocaine-induced reinstatement of seeking behavior. A two-way ANOVA for repeated measures for animals previously self-administered cocaine for tissue level of endocannabinoids and NAEs and expression of cannabinoid receptor experiments showing the significant effect of lever × session interaction ($F(24,336) = 10.72$; $p < 0.001$ and $F(24,336) = 9.47$; $p < 0.001$, respectively). The post hoc analyses revealed a greater frequency of presses on the "active" lever than on the "inactive" lever from the 1st or 2nd cocaine self-administration session till the 1st or 2nd extinction day. During extinction training, neither drug nor drug-paired stimuli were given in response to lever pressing, resulting in a gradual decrease in "active" lever presses ($p < 0.001$). Moreover, an intraperitoneal injection of cocaine induced a significant increase of "active" lever presses during the reinstatement of drug seeking in rats that previously self-administered cocaine ($p < 0.01$). There was no alteration in inactive lever presses after exposure to priming.

In the yoked cocaine and yoked saline groups, the difference between pressing the "active" versus the "inactive" lever failed to reach significance both in the experiment used to evaluate the endocannabinoid and NAEs tissue level and in the expression of cannabinoid receptors (a two-way ANOVA for repeated measures: Yoked cocaine + i.p. cocaine: $F(24,336) = 0.83$; $p = 0.69$ and $F(24,288) = 1.27$; $p = 0.18$; Yoked saline + i.p. cocaine: $F(24,336) = 0.59$; $p = 0.79$ and $F(24,288) = 0.31$; $p = 0.99$; Yoked saline + i.p. saline: $F(24,336) = 0.45$; $p = 0.83$ and $F(24,288) = 0.31$; $p = 0.99$, respectively).

2.2. Tissue Level of Endocannabinoid and NAEs in Rat Brain Structures

In the yoked saline + i.p. saline group, the AEA levels ranged from 11.37 to 16.33 ng/g, with the highest concentration observed in the nucleus accumbens and the lowest in the frontal cortex. As shown in Figure 2, reinstatement of seeking behavior in the animals which had undergone a prior procedure of cocaine self-administration resulted in a changes in the AEA levels in all of the analyzed brain structures: the cerebellum ($F(3,30) = 12.52$; $p < 0.001$), nucleus accumbens ($F(3,28) = 23.41$; $p < 0.001$), hippocampus ($F(3,30) = 6.43$; $p < 0.01$), dorsal striatum ($F(3,29) = 4.88$; $p < 0.05$), frontal cortex ($F(3,28) = 6.70$; $p < 0.05$), and prefrontal cortex ($F(3,28) = 12.46$; $p < 0.001$). A significant decrease in the AEA level was observed in the cerebellum and nucleus accumbens in the animals which self-administered (at least $p < 0.01$) or passively received ($p < 0.001$) cocaine in comparison to the yoked saline animals. Instead, in the prefrontal cortex ($p < 0.01$) and hippocampus ($p < 0.05$) an increase was reported

only in cocaine self-administered rats. Moreover, the animals that had previously self-administered cocaine showed a reduction of the AEA level in the dorsal striatum and frontal cortex when compared to the yoked saline + i.p. cocaine and yoked cocaine + i.p. cocaine rats which, during restatement of seeking-behavior, received an intraperitoneal injection of cocaine (10 mg/kg).

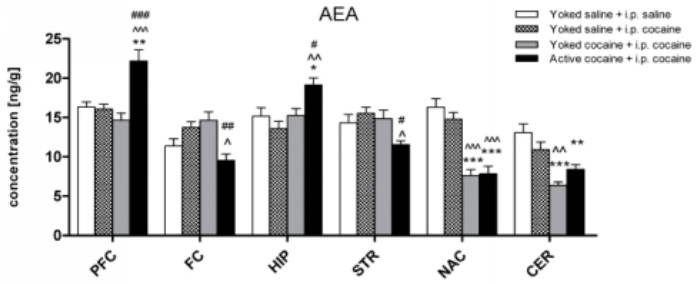

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Figure 2

Levels of anadamide (AEA) in brain structures in rats previously self-administered cocaine (14 days) (Active cocaine) or which passively received cocaine (Yoked cocaine) or saline (Yoked saline) and underwent extinction training (10 days) and drug-induced reinstatement (10 mg/kg cocaine i.p. or saline i.p.). N = 7–8 rats/group. All data are expressed as the means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Yoked saline + saline i.p.; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ vs. Yoked saline + cocaine i.p.; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. Yoked cocaine + cocaine i.p. PFC —prefrontal cortex; FC—frontal cortex, HIP—hippocampus, STR—dorsal striatum, NAC—nucleus accumbens, CER—cerebellum.

The concentration of 2-AG in the control (yoked saline + i.p. saline) group ranged from 3.21 to 5.12 μg/g, with the highest concentration in the dorsal striatum and the lowest in the nucleus accumbens. Cocaine-induced reinstatement influenced the 2-AG levels in the following structures: the frontal cortex ($F(3,28) = 11.27$; $p < 0.001$), hippocampus ($F(3,29) = 33.38$; $p < 0.001$), dorsal striatum ($F(3,30) = 26.22$; $p < 0.001$), nucleus accumbens ($F(3,29) = 3.65$; $p < 0.05$), and cerebellum ($F(3,28) = 6.23$; $p < 0.01$), but not in the prefrontal cortex ($F(3,29) = 2.74$). The post hoc analyses revealed a significant decrease in the 2-AG level in the frontal cortex ($p < 0.001$) and cerebellum ($p < 0.05$), whilst in the hippocampus ($p < 0.001$) and in the nucleus accumbens ($p < 0.05$), an increase was reported in rats that had self-administered cocaine in comparison to the corresponding yoked saline + i.p. saline group. In the animals passively administered cocaine, a significant enhancement in the 2-AG level in the dorsal striatum ($p < 0.001$) and a significant reduction in the cerebellum ($p < 0.05$) was observed (Figure 3).

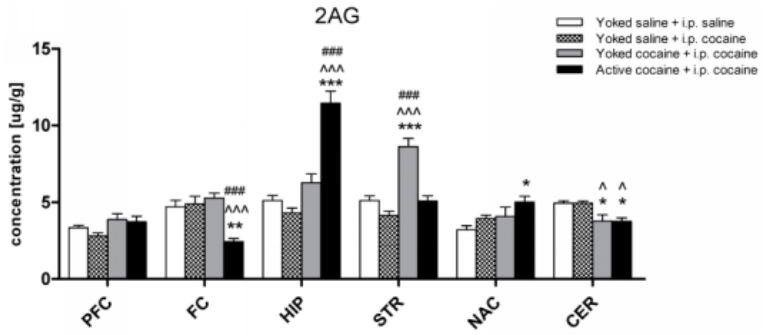

Figure 3

Levels of 2-arachidonoylglycerol (2-AG) in brain structures in rats previously self-administered cocaine (14 days) (Active cocaine) or which passively received cocaine (Yoked cocaine) or saline (Yoked saline) and underwent extinction training (10 days) and drug-induced reinstatement (10 mg/kg cocaine i.p. or saline i.p.). N = 7–8 rats/group. All data are expressed as the means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Yoked saline + saline i.p.; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ vs. Yoked saline + cocaine i.p.; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. Yoked cocaine + cocaine i.p. A description of the abbreviations has been placed under Figure 2.

In the yoked saline + i.p. saline group, the OEA levels ranged from 21.78 to 58.78 ng/g, with the highest concentration in the nucleus accumbens and the lowest in the prefrontal cortex. Cocaine treatment during reinstatement of seeking behavior resulted in a change in the OEA level in the all studied structures: the prefrontal cortex ($F(3,30) = 24.36$; $p < 0.001$), frontal cortex ($F(3,30) = 3.82$; $p < 0.05$), hippocampus ($F(3,30) = 29.15$; $p < 0.001$), dorsal striatum ($F(3,29) = 53.84$; $p < 0.001$), nucleus accumbens ($F(3,30) = 69.02$; $p < 0.001$), and cerebellum ($F(3,29) = 20.80$; $p < 0.001$). In the rats that had self-administered cocaine and those given yoked cocaine injections, a significant increase in the OEA levels was observed in the prefrontal cortex ($p < 0.001$) and dorsal striatum ($p < 0.001$) when compared to the yoked saline + i.p. saline rats. On the other hand, both groups previously administered cocaine, actively or passively, demonstrated a significant reduction of the level of OEA in the hippocampus ($p < 0.001$) and cerebellum ($p < 0.001$). Moreover, the animals previously self-administered cocaine displayed a reduction of the OEA level in the nucleus accumbens ($p < 0.001$) in comparison to all of the yoked groups. The Dunnett's Multiple Comparison test also revealed a significant decrease of the OEA level in the hippocampus of the rats which had previously received saline and were exposed to noncontingent reinforcements ($p < 0.001$) when compared to the corresponding yoked saline + i.p. saline group (Figure 4).

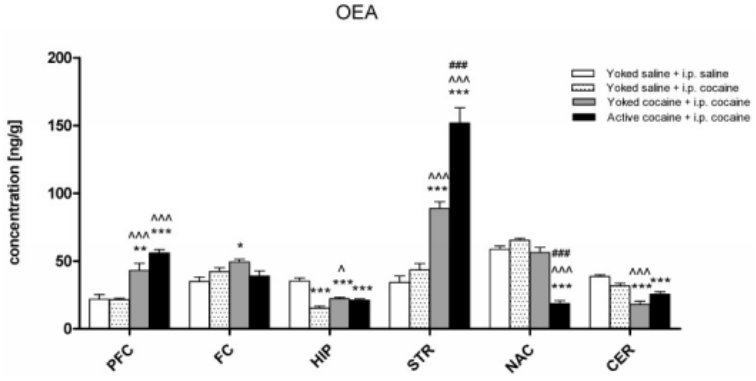

Figure 4

Levels of oleoylethanolamide (OEA) in brain structures in rats previously self-administered cocaine (14 days) (Active cocaine) or which passively received cocaine (Yoked cocaine) or saline (Yoked saline) and underwent extinction training (10 days) and drug-induced reinstatement (10 mg/kg cocaine i.p. or saline i.p.). N = 7–8 rats/group. All data are expressed as the means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Yoked saline + saline i.p.; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ vs. Yoked saline + cocaine i.p.; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. Yoked cocaine + cocaine i.p. A description of the abbreviations has been placed under Figure 2.

The concentration of PEA ranged from 30.90 to 83.60 ng/g in the control (yoked saline + i.p. saline) group, with the highest concentration in the nucleus accumbens and the lowest in the prefrontal cortex. Cocaine administration induced changes in the PEA levels in all of the structures (the prefrontal cortex ($F(3,30) = 231.20$; $p < 0.001$), frontal cortex ($F(3,30) = 59.06$; $p < 0.001$), hippocampus ($F(3,29) = 6.59$; $p < 0.001$), dorsal striatum ($F(3,29) = 68.15$; $p < 0.001$), nucleus accumbens ($F(3,30) = 75.41$; $p < 0.001$), and cerebellum ($F(3,29) = 81.28$; $p < 0.001$)). During cocaine-induced reinstatement in rats previously self-administered cocaine, an increase in the PEA levels was observed in the prefrontal cortex ($p < 0.001$), the hippocampus ($p < 0.05$), and dorsal striatum ($p < 0.001$), while a decrease was noted in the nucleus accumbens ($p < 0.001$) and cerebellum ($p < 0.001$). In rats passively administered cocaine, increases in the PEA levels in the frontal cortex ($p < 0.001$) and dorsal striatum ($p < 0.001$) and decreases in the cerebellum ($p < 0.001$) were reported (Figure 5).

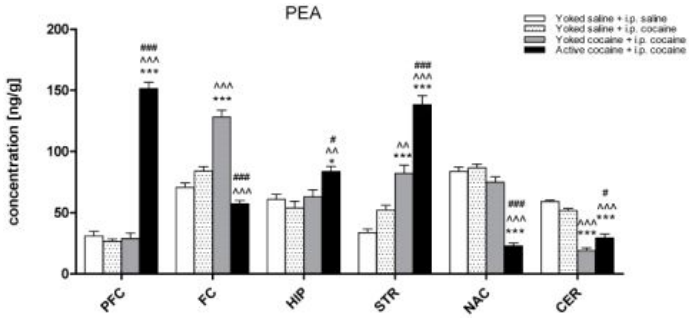

Figure 5

Levels of palmitoylethanolamide (PEA) in brain structures in rats previously self-administered cocaine (14 days) (Active cocaine) or which passively received cocaine (Yoked cocaine) or saline (Yoked saline) and underwent extinction training (10 days) and drug-induced reinstatement (10 mg/kg cocaine i.p. or saline i.p.). N = 7–8 rats/group. All data are expressed as the means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Yoked saline + saline i.p.; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ vs. Yoked saline + cocaine i.p.; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. Yoked cocaine + cocaine i.p. A description of the abbreviations has been placed under Figure 2.

2.3. Expression of the CB1 and CB2 Receptor Protein

In the cocaine-induced reinstatement phase, we observed a decrease in the expression of CB1 receptor protein in the ventral tegmental area ($F(3,24) = 4.95$; $p < 0.01$) in both of the studied groups and in the control group with a cocaine i.p. dose. Furthermore, significant changes were observed in the lateral septal nuclei ($F(3,24) = 8.54$; $p < 0.001$) and prefrontal cortex ($F(2,14) = 8.46$; $p < 0.01$), and an increase of the expression of CB1 receptors was observed in the group of rats that had actively self-administered cocaine (at least $p < 0.05$). Interestingly, significant changes were demonstrated in the dorsal striatum ($F(3,24) = 4.93$; $p < 0.05$), and a decrease in the CB1 receptors level was observed in the yoked cocaine + i.p. cocaine group. A one-way ANOVA did not show changes in CB1 receptor expression in the basolateral and basomedial amygdala ($F(3,24) = 1.06$), nucleus accumbens core ($F(3,24) = 2.43$) and shell ($F(3,24) = 0.46$), medial globus pallidus ($F(3,24) = 1.69$), hippocampus ($F(3,24) = 1.23$), and substantia nigra ($F(3,24) = 2.72$). The results of the analysis are shown in Figure 6 (top panel).

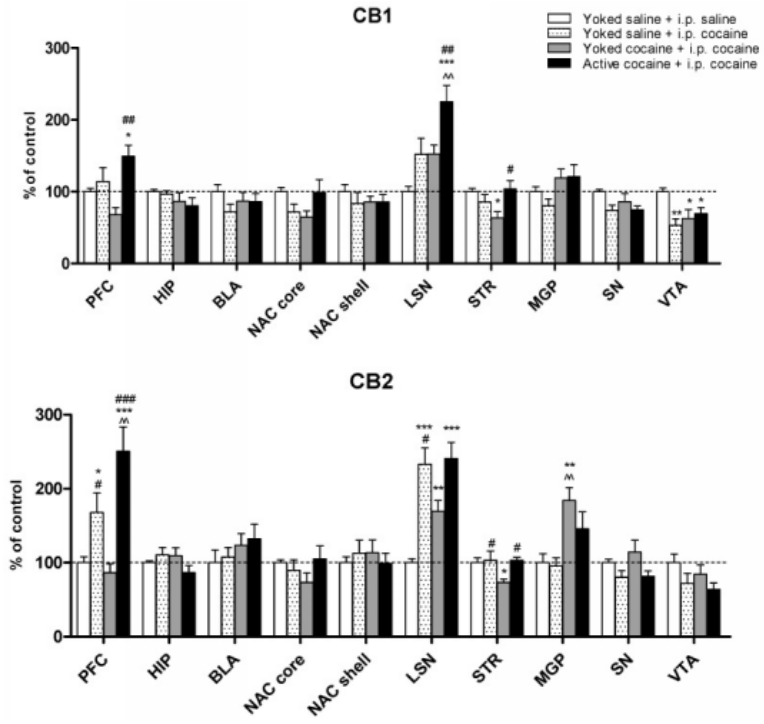

Figure 6

Results of immunohistochemistry analysis of membrane's cannabinoid receptor 1 and 2 in the reinstatement cocaine phase. All data are expressed as the means ± SEM. N = 7 rats/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Yoked saline + saline i.p.; ^^ $p < 0.01$ vs. Yoked saline + cocaine i.p.; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ $^{\#\#\#}$ $p < 0.001$ vs. Yoked cocaine + cocaine i.p. PFC—prefrontal cortex; HIP – hippocampus, BLA—basolateral and basomedial amygdala, NAC core or shell—nucleus accumbens core or shell, LSN—lateral septal nuclei, STR—dorsal striatum, MGP—medial globus pallidus SN—substantia nigra, VTA—ventral tegmental area.

In the CB2 receptor expression, we noticed statistically significant changes in the lateral septal nuclei ($F(3,24) = 9.85$; $p < 0.001$), and the post hoc

tests demonstrated an increase in the receptor level in chronic and acute cocaine-treated rats ($p < 0.001$). Moreover, significant changes were noted in the prefrontal cortex ($F(3,24) = 11.39$; $p < 0.001$), and an increase in the receptors was noticed in active cocaine self-administered ($p < 0.001$) and yoked saline + i.p. cocaine ($p < 0.05$) animals exposed to cocaine intraperitoneal injection during reinstatement. Furthermore, significant changes were demonstrated in medial globus pallidus ($F(3,24) = 6.53$; $p < 0.01$), while an increase was observed only in yoked cocaine + i.p. cocaine rats ($p < 0.01$). Also in the dorsal striatum a significant changes in the CB2 receptor expression were indicated (one-way ANOVA ($F(3,24) = 3.57$; $p < 0.05$); a decrease in the receptors level in the passive cocaine group was noted in comparison to the other experimental groups. In the nucleus accumbens core ($F(3,24) = 1.14$), nucleus accumbens shell ($F(3,24) = 0.29$), basolateral and basomedial amygdala ($F(3,24) = 0.80$), the substantia nigra ($F(3,24) = 2.58$), hippocampus ($F(3,24) = 1.60$), and ventral tegmental area ($F(3,24) = 1.77$), the level of CB2 receptors was unchanged. The results of analysis are shown in Figure 6 (bottom panel).

Table 1 shows the observed changes in tissue levels of eCS and NAEs and changes in the expression of cannabinoid receptors (CB1 and CB2) in the rat brain structures.

Table 1

Changes in the endocannabinoid, NAE (tissue levels), and cannabinoid receptors (CB1 and CB2) in the selected rat brain structures during cocaine-induced reinstatement.

Brain Structure	AEA			2-AG			OEA			PEA			CB1			CB2		
	A	Y	YS	A	Y	YS	A	Y	YS	A	Y	YS	A	Y	YS	A	Y	YS
PFC	↑	--	--	--	--	--	↑	↑	--	↑	--	--	↑	--	--	↑	--	↑
FC	--	--	--	↓	--	--	--	↑	--	--	↑	--	--	--	--	--	--	--
HIP	↑	--	--	↑	--	--	↓	↓	↓	↑	--	--	--	--	--	--	--	--
BLA						n/a							--	--	--	--	--	--
NAC core	↓	↓	--	↑	--	--	↓	--	--	↓	--	--	--	--	--	--	--	--
NAC shell													--	--	--	--	--	--
LSN						n/a							↑	--	--	↑	↑	↑
STR	--	--	--	--	↑	--	↑	↑	--	↑	↑	--	--	↓	--	--	↓	--
MGP													--	--	--	--	↑	--
SN						n/a							--	--	--	--	--	--
VTA													↓	↓	↓	--	--	--
CER	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓	--			n/a			

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A—active cocaine + i.p. cocaine group, Y—yoked cocaine + i.p. cocaine group; YS—yoked saline + i.p. cocaine. PFC—prefrontal cortex; FC—frontal cortex, HIP—hippocampus, BLA—basolateral amygdala, NAC core and shell—nucleus accumbens core and shell, LSN—lateral septal nuclei, STR—dorsal striatum, MGP—medial globus pallidus, SN—substantia nigra, VTA—ventral tegmental area, CER—cerebellum. ↑—increase, ↓—decrease, ---—no changed, n/a—not analyzed.

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3. Discussion

The present study demonstrates the alterations in the level of endocannabinoids and cannabinoid CB1 and CB2 receptors in the rat brain structures following a cocaine-induced relapse. At the same time, we also found cocaine-induced changes in OEA and PEA concentrations after the cocaine challenge dose in the animals with a previous motivational and experimental delivery of cocaine. This paper follows our previous study where we reported several changes in the concentration of AEA and 2-AG as well as OEA and PEA linked to cocaine reward or to cocaine withdrawal [2].

In the current study, a cocaine priming dose resulted in a significant increase of the AEA level in the hippocampus and prefrontal cortex only in animals with a previous motivational intake of cocaine. Since the changes were not present in yoked saline + i.p. cocaine or cocaine + i.p. cocaine controls (present study), during cocaine self-administration and in the cocaine-free period with extinction training [2], it means the specific effect of a cocaine-induced relapse is attributed to the limbic AEA levels. The reductions of the AEA levels in the frontal cortex and striatum linked to previous cocaine self-administration and in the nucleus accumbens and cerebellum due to repeated cocaine injections did not reflect cocaine-induced reinstatement but, instead, rather persistent adaptations after chronic cocaine exposure [2].

With regards to the second endocannabinoid studied, the 2-AG levels also increased in the hippocampus and nucleus accumbens while they decreased in the frontal cortex only after cocaine priming in rats previously self-administered cocaine. The recent literature data confirms the involvement of the endocannabinoid molecules (especially 2-AG) in the hippocampus in the (i) modulation of the dopaminergic system [17,18] and in (ii) cocaine-induced reinstatement [19]. In fact, we reported that systemic injections with blockers of fatty acid amide hydrolase (FAAH), the main enzyme responsible for terminating the actions of the endocannabinoid anandamide, was able to reduce a cocaine-primed relapse in rats [20]. The protective effects of FAAH inhibitions were also noted for cocaine seizures and cell death in the hippocampus in a model of the drug's intoxication in mice [21]. Furthermore, previous research on the endocannabinoids and stress responses indicated a significant role of 2-AG in regulating stress behaviors [22]. In chronic stress, the 2-AG level increased [23], and the rise in the striatal 2-AG level in may indicate adaptation to a stressful situation in the rats that had had a passive cocaine intake.

Parallel immunohistochemical analyses revealed that cocaine priming enhanced the expression of the CB1 and CB2 receptors in the prefrontal cortex and lateral septal nuclei. The upregulation of the CB1 receptors observed in the prefrontal cortex and the lateral septal nuclei was present during cocaine self-administering rats and should be linked to the drug rewarding properties [7]. The lateral septum is the middle of the relay for connections from the CA3 hippocampus to the ventral tegmental area, which makes it possible to link the reward signals with the context in which they occur [4]. An increase in the expression of CB1 and CB2 receptors observed in the lateral septal nuclei may also be the result of conditional stimuli accompanying the experiment (sound and light signal associated with the dose of the drug) [24]. The present neurochemical data supports our previous behavioral findings in which we reported that tonic activation of CB1 is involved in cocaine-induced reinstatement of cocaine-seeking behavior [20]. Interestingly, we also observed a decrease in the CB1 receptor expression in the ventral tegmental area seen in all of the studied rat groups, including the animals treated with acute cocaine. Reduced levels of CB1 receptors in the ventral tegmental area in all of the experimental groups suggest a pharmacological effect of cocaine-priming. In the ventral tegmental area, CB1 receptors are expressed on GABA neurons and their

reduction observed in this paper suggests a pharmacological effect of cocaine-priming leading to the rise of the dopamine level and increased sensitivity to the drug effects [3,25,26]. Upregulation of the CB2 receptors seems to be related to the last cocaine dose, because an increase in the CB2 receptor expression was also presented in the control group with a single i.p. cocaine dose.

The levels of endocannabinoids reflect their concentration at the time of tissue collection for analysis (the neurotransmitter presence in the synaptic space and in presynaptic neurons), which may not necessarily correlate with the expression of cannabinoid receptors. Similar results were reported during chronic stress, where the 2-AG level was increased [23], while the expression of CB1 receptor did not change in any brain regions [27].

Recently, Luchicchi et al. (2010) demonstrated that NAEs play a role in behavioral and neurochemical effects related to cocaine addiction [28]. The finding from this study showed that NAEs are involved in the relapse of cocaine-seeking behavior. OEA and PEA are endogenous lipid transmitters with similar properties to AEA and 2-AG, although they did not act on cannabinoid receptors. They act through the nuclear proliferator-activated receptor alpha (PPARα), the transient receptor potential vanilloid 1 (TRPV1), and the orphan G-protein-coupled receptors GPR55 and GPR119. NAEs participate in the regulation of many physiological processes and they have neuroprotective, antinociceptive, and anti-inflammatory effects [29]. In this paper, we found that a cocaine-induced relapse resulted in a potent increase in OEA levels in the prefrontal cortex and striatum and in PEA in the prefrontal cortex, hippocampus and dorsal striatum. At the same time, there were potent reductions in the NAEs level in the rat cerebellum. The above changes were noted for chronic cocaine experienced rats, while a decrease in the tissue levels of OEA and PEA observed in the nucleus accumbens was linked with cocaine relapse in rats. Decreased levels of OEA and PEA in nucleus accumbens after the cocaine challenge only in rats self-administering cocaine suggest an impaired control of the release of neurotransmitters during reinstatement, and correlate with the previously reported the participation of the TRPV1 receptor [13,20,28]. Recently, Zambrana-Infantes et al. (2018) demonstrated that repeated administration of PEA can block behavioral cocaine sensitization in the mechanism, probably independent of the PPAR-α receptor, but rather as a result of the anti-inflammatory properties of PEA [29]. The decrease in PEA and OEA in the nucleus accumbens observed in our study may confirm the increased cocaine sensitization as a result of the habit forming, since the same changes have not been observed in cocaine-experienced animals following repeated passive (yoked) treatment. It is also worth noting that acute administration of OEA reduced cocaine locomotion or reward but did not affect cocaine sensitization, conditioned place preference, and reinstatement to cocaine in PPARα receptor knockout in mice [30]. The decrease in PEA and OEA in nucleus accumbens observed in our study may confirm the increased cocaine sensitization after a cocaine-priming dose as a result of the habit forming, but pharmacological analyses with selective ligands are required.

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4. Materials and Methods

4.1. Animals

Male Wistar rats (280–300 g, N = 70) delivered by a licensed breeder (Charles River, Germany) were housed individually in standard plastic rodent cages in a colony room maintained at 22 ± 2 °C and at 45–65% humidity under a 12-h light–dark cycle (lights on at 06:00). The animals had free access to standard animal food and water during the 7-day habituation period. We used a yoked procedure to assess whether a motivational factor would have an impact. Then, the animals were divided into four groups: active cocaine + i.p. cocaine, yoked cocaine+ i.p. cocaine, yoked saline + i.p. cocaine, and yoked saline + i.p. saline and those used in the cocaine self-administration procedures were maintained on limited water during the initial training sessions (see below). All of the experiments were conducted during the light phase of the light–dark cycle (between 8.00 a.m. and 3.00 p.m.) and the experiments were carried out in accordance with the European Directive 2010/63/EU and approved by the Ethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow. All of the animals used in the study were experimentally naïve.

4.2. Drugs

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), dissolved in sterile 0.9% NaCl and given iv (0.1 mL/infusion) or i.p. (1 mL/kg).

4.3. Behavioral Procedures

4.3.1. Cocaine Self-Administration, Extinction Training and Reinstatement of Drug-Seeking Behavior

After a week of habituation to the animal facility, animals were water-deprived for 18 h and subsequently trained to press the lever of standard operant conditioning chambers (Med-Associates, ST Albans City, VT, USA) under an increasing schedule of water reinforcement at a fixed ratio (FR) of 1 to 5. Two days following “lever-press” training under FR5 and free access to water, the rats were chronically implanted with a silastic catheter in the external right jugular vein, as described previously [31]. The catheters were flushed every day with 0.1 mL of saline solution containing heparin (70 U/mL, Biochemie GmbH, Kundl, Austria) and 0.1 mL of solution of cephazolin (10 mg/mL; Biochemie GmbH, Kundl, Austria).

After a 7–8-day recovery period, all of the animals were water deprived for 18 h and trained to lever press to a FR5 schedule of water reinforcement over a 2-h session. Some of the subjects (active cocaine groups) were then given access to cocaine during 2-h daily sessions performed 6 days/week (maintenance) and from that time they were given ad libitum water. The house light was illuminated throughout each session. Each completion of five presses on the “active” lever complex (the FR5 schedule) resulted in a 5-s infusion of cocaine (0.5 mg/kg per 0.1 mL) and a 5-s presentation of a stimulus complex (activation of the white stimulus light directly above the “active” lever and the tone generator, 2000 Hz; 15 dB above ambient noise levels). Following each injection, there was a 20-s time-out period during which the responses were recorded but had no programmed consequences. Those on the “inactive” lever did not result in cocaine delivery. Acquisition of the conditioned operant response lasted a minimum of 10 days until the subjects met the following criteria: active lever presses with an average of 3 consecutive days and a standard deviation within those 3 days of <10% of the average; this selected criterion was based on our prior experiments [32]. The animals trained to self-administer cocaine (0.5 mg/kg/infusion) (which met the acquisition criterion) were used in the extinction study and next in the reinstatement test. During extinction training, the subjects had 2-h daily training sessions with no cocaine delivery (saline was substituted for cocaine) and no presentation of the conditioned stimulus. Once they reached the extinction criteria (a minimum of 10 extinction days and with the responses to the active lever below 10% of the level observed during maintenance over at least 3 consecutive days), on the 10th day of extinction, the animals were tested for the response reinstatement induced by a noncontingent presentation of a self-administered reinforcements (10 mg/kg cocaine i.p.). During the reinstatement tests (2-h sessions), active lever presses on the FR5 schedule resulted in intravenous injection of saline. All of the animals were sacrificed after a 2-h experimental session.

4.3.2. Yoked Self-Administration Procedure

The rats were tested simultaneously in groups of three with two rats serving as ‘yoked’ controls that received an injection of saline or cocaine which was not contingent on responding, and each time a response-contingent injection of 0.5 mg/kg cocaine was self-administered by the paired rat. Unlike the self-administering rats, the lever pressing by the yoked rats was recorded but had no programmed consequences [33]. The yoked saline + i.p. saline, saline + i.p. cocaine, and cocaine + i.p. cocaine animals were sacrificed at the same time as the corresponding self-administered cocaine groups of rats after a 2-h experimental session of reinstatement drug seeking. The plan of the experiment is presented

in Figure 7.

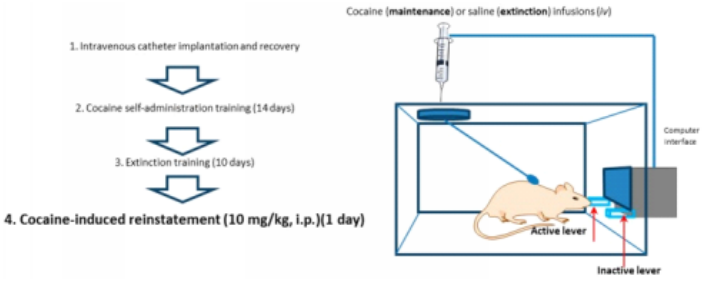
Cocaine (maintenance) or saline (extinction) infusions (iv)
1. Intravenous catheter implantation and recovery
2. Cocaine self-administration training (14 days)
3. Extinction training (10 days)
4. Cocaine-induced reinstatement (10 mg/kg, i.p.)(1 day)
Computer interface
Active lever
Inactive lever

Figure 7

Illustration of cocaine self-administration experiments.

4.4. Neurochemical Analysis

4.4.1. LCMS/MS Analysis

Brain Structures Isolation and Tissue Preparation

The rats were sacrificed through decapitation. Selected brain structures (i.e., the prefrontal cortex, frontal cortex, hippocampus, dorsal striatum, nucleus accumbens, and cerebellum; Figure 8 panel A) were isolated, immediately frozen on dry ice and stored at −80 °C. the tissues were dissected out according to The Rat Brain Atlas and to the chapter in Neuroproteomics [34,35].

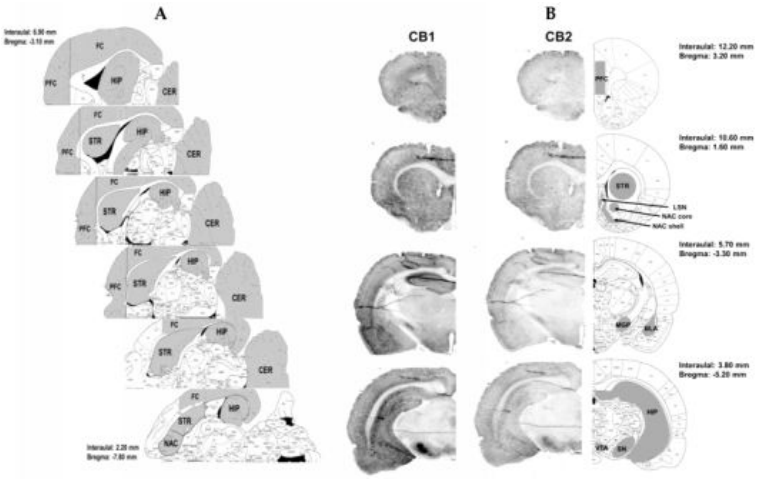

Figure 8

Illustration of rat brain structures for detection of level of endocannabinoid and NAEs (panel **A**; horizontal section) or immunostaining of cannabinoid CB1 and CB2 receptors after passive saline administration, extinction training, and saline injection during reinstatement (Yoked saline + i.p. saline) (panel **B**; coronal sections). BLA—basolateral and basomedial amygdala, CER—cerebellum, FC—frontal cortex, HIP—hippocampus, LSN—lateral septal nucleus, MGP—medial globus pallidus, NAC core—nucleus accumbens core, NAC shell—nucleus accumbens shell, PFC —prefrontal cortex, SN—substantia nigra, STR—dorsal striatum, and VTA—ventral tegmental area. According to the Rat Brain Atlas [34].

Reagents

Standards of AEA, 2-AG, PEA, and OEA were obtained from Tocris (Bristol, United Kingdom), AEA-d_4, 2-AG-d_5, PEA-d_4 and OEA-d_4 were from Cayman Chemical (Ann Arbor, MI, USA), acetonitrile and chloroform were from Merck (Darmstadt, Germany), while methanol and formic acid were from POCh (Katowice, Poland). All the stock solutions of the standards, excluding 2-AG and 2-AG-d_5, were prepared in ethanol, whereas 2-AG and 2-AG-d_5 stock solutions were prepared in acetonitrile (concentration 1 mg/mL). All the stock solutions were stored at −80 °C. Further dilutions were prepared using acetonitrile.

Lipid Extraction from Brain Tissue

The extraction was carried out by a method of lipid compounds' isolation developed by Folch in 1957 with our modifications [36]. The tissues were homogenized in an ice-cold mixture of methanol and chloroform (1:2, *v*/*v*) at the proportion of 10 mg of wet tissue per 150 µL of solvent. Next, 150 µL of the homogenate was mixed with 250 µL of formic acid (pH 3.0; 0.2 M) and 1500 µL of the extraction mixture (methanol: chloroform; 1:2, *v*/*v*). Then, 2 µL of an internal standard (AEA-d4, at 10 µg/mL; 2-AG-d_5 at 100 µg/mL, and PEA-d_4, OEA-d_4 at 5 µg/mL) were added to each sample. The samples were afterwards vortexed for 30 s and centrifuged at 2000 rpm for 10 min. The organic phases were collected and dried under a nitrogen stream at 40 °C. The dry residue was dissolved in 40 µL of acetonitrile and 10 µL of the reconstituted extract was injected into the LC-MS/MS system for a quantitative analysis purpose.

Chromatographic Analysis: LC-MS/MS Conditions

The LC-MS Applied Biosystem API 2000 (Perlan Technologies, Warsaw, Poland) instrumentation consisted of the following components: an Agilent series 1100 pump (Santa Clara, CA, USA) in its gradient mode and an autosampler Agilent series 1100 with a 100 µL injection loop.

The chromatographic separation was performed in the gradient mode with a Thermo Scientific BDS HYPERSIL (Waltham, MA, USA) C18 column 100 × 3 mm I.D., 3 µm particle size. The advance column, with a precolumn (10 × 3 mm I.D., 3 µm particle size), was maintained at 40 °C and the mobile phase flow rate was 0.3 mL/min. The mobile phases for gradient elution consisted of A (formic acid (0.02 M) in acetonitrile) and B (formic acid (0.02 M) in water). The gradient started initially at 0% A over 1 min, then increased linearly to 90% by 2 min, where it stayed for the next 2 min; before the solvent A content decreased to 0% over 6 min. Finally, for the last 4 min of the analysis, the column was maintained at 100% B to stabilize the baseline. The sample temperature was maintained at 4 °C in the autosampler prior to analysis.

Quantification of the analytes was performed using the tandem electrospray MS in a positive mode (ESI). The data acquisition and processing were accomplished using the Applied Biosystems Analyst version 1.4.2 software. Endocannabinoid and NAE concentrations in the samples were calculated with the calibration curve prepared using the brain tissues samples collected from naive rats (rats not previously subjected to experimentation, without a particular experimental situation and without a particular drug/antigen used before) to eliminate the matrix effect. The brain tissues samples were spiked with AEA, PEA, and OEA to obtain the concentration of 0.2; 2; 20; 50; 100; 200 ng/g and with 2-AG to obtain the concentration of 0.8; 2; 10; 20; 50; 100 µg/g. AEA-d_4, 2-AG-d_5, PEA-d_4 and OEA-d_4 were used as the internal standard. The procedure described for

the sample preparation was followed for sample analysis.

The endocannabinoid levels were determined from the calibration curve by means of linear regression applied to the response ratios (peak area for specific analyte/peak area for the internal standard) as a function of the corresponding endocannabinoid/NAE concentration. To ensure the method was sufficiently thorough, analytical assessments for selectivity, linearity, precision, accuracy, recovery, and stability were performed [36].

4.4.2. Immunohistochemistry

The rats were sacrificed immediately after the experimental session with pentobarbital and perfused intracardially with a solution of 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) in 100 mM phosphate buffer, pH 7.4 (PBS; 81 mM Na_2HPO_4, 19 mM NaH_2PO_4, 27 mM KCl, 154 mM NaCl; POCH, Gliwice, Poland). The brains were excised and post-fixed in the same fixative for 12 h. The tissue was permeated with in 10% *w*/*v* sucrose (Sigma Aldrich, St. Louis, MO, USA) for 7 days and removed in 30% *w*/*v* sucrose in PBS at 4–8 °C for no less than 48 h. The brains were deeply frozen on dry ice and cut into 12 µm coronal sections on a cryostat (Leica Microsystems, Nussloch, Germany) and were kept at −20–−22 °C until processed for immunohistochemistry.

Immunohistochemistry Procedure

The rat brain sections were rinsed in 100 mM PBS buffer pH 7.5, and in PBS containing 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), for 30 min at room temperature and Odyssey Blocked Buffer (OBB; Li-COR Biosciences, Cambridge, UK) for 1h at room temperature. The tissue sections were incubated overnight at 4 °C with purified primary antibodies in OBB containing 0.1% Tween 20: 1:200 dilution of rabbit monoclonal anti-CB_1 receptor (Abcam, ab172970), and 1:200 dilution of goat polyclonal anti-CB2 (Santa-Cruz, sc-10076; [37]). Following four washes of 5 min each with PBS containing 0.1% Tween 20, the tissue sections were incubated for 1 h at RT with goat anti-rabbit (IRDye® 680CW, Li-COR Biosciences, Cambridge, UK) and donkey anti-goat (IRDye® 800CW, Li-COR Biosciences, Cambridge, UK) secondary antibodies at a dilution of 1:2000. Fluorescence was detected using the Odyssey® Infrared Imaging System (21 µm resolution, 1 mm offset with highest quality) (Li-COR, Lincoln, NE, USA). Channel sensitivity was optimized for each set of stained sections and maintained for that group of samples. The relative location of the slice and identification of brain regions were determined by comparison to images in the Paxinos and Watson Rat Brain Atlas (2007) [7] (Figure 8 panel B).

4.5. Data Analysis

Animals that had problems with catheters during the recovery period, not performing the self-administered acquisition and extinction criteria or those showing incorrect perfusion were excluded from the data analysis. All the data were expressed as means (± SEM). Behavioral data were analyzed by a two-way ANOVA for repeated measures and for statistical evolution of these data across the following factors: lever ("active" and "inactive") and sessions where self-administration, extinction training, and reinstatement of seeking behavior were used. Significant group differences according to an ANOVA were analyzed by the post hoc Newman–Keuls test. Neurochemical data were analyzed by one-way ANOVA for different treatment groups (Yoked saline + i.p. saline, Yoked saline + i.p. cocaine, Yoked cocaine + i.p. cocaine, and Active cocaine + i.p. cocaine) and appropriate, post hoc comparisons were conducted by a Dunnett's Multiple Comparison test. A p value < 0.05 was considered as statistically significant.

Go to:

5. Conclusions

We found that endocannabinoids (AEA and 2-AG) and endogenous noncannabinoid lipid signaling molecules (PEA, OEA) are engaged in the reinstatement of cocaine-seeking behavior in rats. Our results support behavioral analyses with selective cannabinoid, TRPV1 or PPAR-α receptor ligands to control cocaine addiction. The present findings extend our previous observations that the endocannabinoid and NAE molecules and CB1 receptors influence cocaine self-administration.

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Author Contributions

Conceptualization, M.F. (Małgorzata Filip); Formal analysis, B.B. and I.S.; Investigation, B.B. and M.F. (Małgorzata Frankowska); Methodology, B.B. and M.F. (Małgorzata Frankowska); Project administration, M.F. (Małgorzata Filip); Validation, B.B.; Visualization, B.B.; Writing—original draft, B.B., E.N.-A. and L.P.-C.; Writing—review & editing, M.F. (Małgorzata Filip).

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Conflicts of Interest

The authors declare no conflict of interest.

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Highlights

AEA and 2-AG play a role in cocaine-induced seeking behavior.

A drug-induced relapse induces up- and downregulation of brain cannabinoid receptors in rats.

Cocaine priming evokes bidirectional changes in the OEA and PEA in the rat brain.

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Footnotes

Sample Availability: Not available.

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Circulating Endocannabinoids: From Whence Do They Come and Where are They Going?

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Abstract

The goal of this review is to summarize studies in which concentrations of circulating endocannabinoids in humans have been examined in relationship to physiological measurements and pathological status. The roles of endocannabinoids in the regulation of energy intake and storage have been well studied and the data obtained consistently support the hypothesis that endocannabinoid signaling is associated with increased consumption and storage of energy. Physical exercise mobilizes endocannabinoids, which could contribute to refilling of energy stores and also to the analgesic and mood-elevating effects of exercise. Circulating concentrations of 2-arachidonoylglycerol are very significantly circadian and dysregulated when sleep is disrupted. Other conditions under which circulating endocannabinoids are altered include inflammation and pain. A second important role for

endocannabinoid signaling is to restore homeostasis following stress. Circulating endocannabinoids are stress-responsive and there is evidence that their concentrations are altered in disorders associated with excessive stress, including post-traumatic stress disorder. Although determination of circulating endocannabinoids can provide important information about the state of endocannabinoid signaling and thus allow for hypotheses to be defined and tested, the large number of physiological factors that contribute to their circulating concentrations makes it difficult to use them in isolation as a biomarker for a specific disorder.

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Introduction

Although the last several centuries have brought fundamental improvements in Western societies that have resulted in significant benefits to human health, these improvements have been accompanied by adverse changes in food intake, sleep patterns, and exposure to psychological stress that contribute to risk for obesity, diabetes, anxiety and depression. Accumulating evidence from preclinical studies demonstrates that the endocannabinoid signaling system is involved in all of these diseases and disorders; hyperactivity of endocannabinoid signaling contributes to excessive intake and storage of high-calorie foods, while endocannabinoid hypoactivity is a risk factor for the development of depression, anxiety and post-traumatic stress disorder. Millennia of human use of cannabis sativa, which contains the endocannabinoid mimetic Δ^9-tetrahydrocannabinol (THC), and a brief period in which humans were treated with the endocannabinoid antagonist, rimonabant, provide support for the hypothesis that endocannabinoid signaling is similarly involved in humans. Thus, better understanding of the endocannabinoid system in humans is an important step toward more sophisticated exploitation of this system to further improve human health.

Assessment of circulating endocannabinoids has been an important but sometimes frustrating approach to the assessment of endocannabinoid signaling in humans. The endocannabinoids are present in the human circulation and their concentrations are dynamic. The purpose of this review is to compile the available data that shed light on several important questions about endocannabinoids that are in the circulation, including: What physiological and pathophysiological factors regulate their concentrations? What are their sources? What are their physiological or pathological roles? Do relative concentrations of circulating endocannabinoids provide clinical information; in other words, are they useful biomarkers?

The available data indicate that circulating endocannabinoids come from multiple organs and tissues, including brain, muscle, adipose tissue and circulating cells. There are hints that endocannabinoids are ‘placed’ into the circulation to accomplish a specific purpose, as in the regulation of energy intake following exercise. In other cases, it seems more likely that endocannabinoids enter the circulation by virtue of the fact that they are highly lipophilic and can equilibrate from organs and tissues into the circulation, in other words, are an indirect marker of tissue endocannabinoid tone. The available data also provide some hints and hypotheses regarding the targets of the circulating endocannabinoids, including the metabolic organs and the brain. The key sources and targets supported by data obtained from endocannabinoid measurements in human serum and plasma are shown in Figure 1.

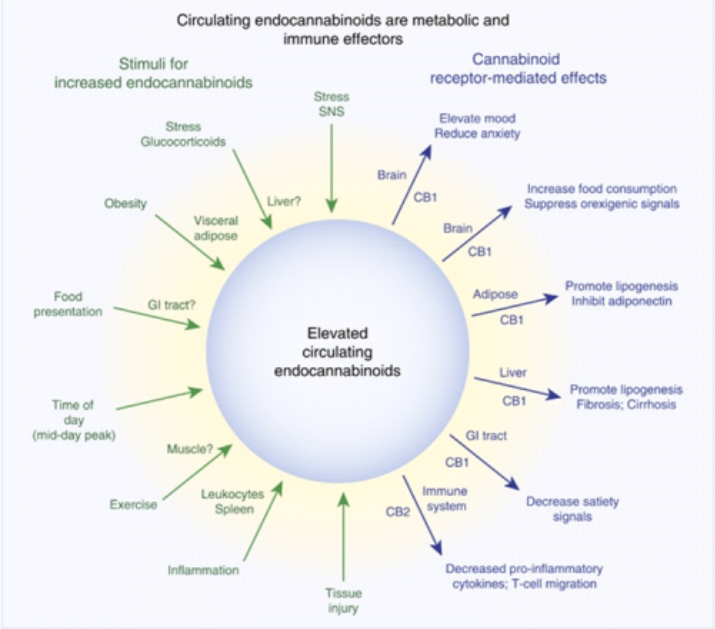

Figure 1

Schematic representing, on the left side, the stimuli and potential sources of endocannabinoids that are present in the circulation and, on the right side, the potential targets of endocannabinoids, together with the cannabinoid receptor subtype that is involved. These concepts are supported by the citations and discussions throughout the review.

The Endocannabinoids

The two most well-studied endocannabinoids (defined as endogenous ligands for cannabinoid receptors) are the arachidonic acid derivatives, *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). The endocannabinoids are found in all tissues, organs and bodily fluids thus far investigated. Both AEA and 2-AG are endogenous agonists of the canonical cannabinoid receptors, CB1R and CB2R, G protein coupled receptors that have a widespread distribution throughout the body. In addition to the CB receptors, both endocannabinoids can also bind and activate the vanilloid receptor (TRPV1) and AEA is an agonist for several subtypes of the peroxisome proliferator-activated receptor (PPAR) family.

The biosynthetic and catabolic pathways for both endocannabinoids have been studied and elucidated to varying degrees (Hillard, 2015). 2-AG is produced as a result of activation of phospholipase C (PLC) in cells that express diacylglycerol lipase. Diacylglycerol lipase converts the PLC product diacylglycerol to 2-acylglycerols, including 2-AG and 2-oleoylglycerol (2-OG). The concentration of 2-OG in human serum is typically 5–10 times greater than 2-AG. It has been suggested that 2-OG is an endogenous ligand for the orphan receptor GPR119, a receptor that plays an important role in coupling food intake to the release of incretin hormones (Syed *et al*, 2012).

2-AG is catabolized by hydrolysis of its ester bond, resulting in the formation of glycerol and free arachidonic acid. Several enzymes can accomplish this hydrolysis, including monoacylglycerol lipase (MAGL), alpha-beta hydrolase domain protein (ABHD)-6, and ABHD-12 (Blankman *et al*, 2007). Because it is metabolized to arachidonic acid by MAGL, 2-AG functions as a precursor for the formation of other arachidonic acid derivatives in the vasculature (Gauthier *et al*, 2005) and in the brain (Nomura *et al*, 2008; Viader *et al*, 2015). In the brain, MAGL activity has been shown to play a role in the regulation of neuroinflammation through this mechanism (Viader *et al*, 2015). 2-AG can also serve as a substrate for enzymes that metabolize free arachidonic acid, including cyclooxygenase 2 (COX2) and some subtypes of lipoxygenase (Urquhart *et al*, 2015).

AEA and other *N*-acyl ethanolamines (NAEs) are synthesized from a low abundance phospholipid, *N*-acyl-phosphatidylethanolamine (NAPE), through several different pathways (Hillard, 2015). NAPE is synthesized via enzymatically mediated transfer of an acyl chain from the sn-1 position of a donor phosphatidylcholine to the amine of phosphatidylethanolamine (PE). The relative abundance of the NAEs formed reflects the relative abundance of the acyl chains found in the sn-1 position of the donor phospholipids; thus, concentrations of palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are generally higher than AEA in biological samples, including serum and plasma. PEA and OEA exert anti-inflammatory and anorexigenic effects, respectively likely as a result of interactions with PPARα (Guzman *et al*, 2004). The NAEs are catabolized by hydrolysis of the amide bond through the actions of fatty acid amide hydrolase (FAAH) (Cravatt *et al*, 1996) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), which is found primarily in peripheral tissues (Ueda *et al*, 2001). AEA can also serve as a substrate for COX-2, and lipoxygenase and P450 enzymes that utilize arachidonic acid as a substrate (Urquhart *et al*, 2015).

Measurement of Endocannabinoids in the Circulation

Endocannabinoid concentrations in the circulation are typically measured in lipid extracts of serum or plasma obtained from venous blood using isotope-dilution, liquid chromatography-mass spectrometry. Although the endocannabinoids can be measured in both plasma and serum, there is evidence that AEA concentrations are higher in serum than in plasma (Lam *et al*, 2010) and 2-AG concentrations are more reliably measured in serum (Hillard *et al*, 2012). Known amounts of deuterated analogs of AEA and 2-AG are added to the serum or plasma samples at the beginning of the extraction process. These analogs are chemically indistinguishable from the naturally occurring molecules, yet have a mass that is 1 dalton higher for every deuterium atom that has been incorporated (usually 8) so can be differentiated from their endogenous counterparts in the mass spectral analyses. As a result, the recovery of the deuterated analog can be measured and provides an excellent estimate of recovery of the endogenous compounds through the extraction and separation procedures.

Blood cells can both release and inactivate AEA (Schmidt *et al*, 2006; Varga *et al*, 1998), thus the concentrations of AEA measured in serum or plasma are dependent upon the time between the blood draw and the separation of cells from serum or plasma (Vogeser *et al*, 2006). As a result, it is best practice to keep the time between blood draw and serum or plasma harvest as consistent as possible to minimize between sample differences in *ex vivo* generation or loss of AEA. AEA concentrations are stable in serum or plasma after it has been frozen (Jian *et al*, 2010). The arachidonic acid in 2-AG readily migrates to the 1 position under most storage and extraction conditions (Rouzer *et al*, 2002). 2-AG and 1-AG migrate at different rates in the standard chromatography used to separate the endocannabinoids. Most methods assume that both 2-AG and 1-AG originate from biological 2-AG and add these peaks together to determine total 2-AG.

Several methods have been published for the extraction of endocannabinoids and related lipids from liquid matrices such as serum and plasma (Gouveia-Figueira and Nording, 2014). A liquid-liquid extraction method has been described in which the sample is mixed with methanol and chloroform with repeated vortexing (De Marchi *et al*, 2003). Acetonitrile can also be used as the organic solvent and sonication the method of mixing. Solid phase extraction using C18 columns of the samples that have been made 15% in ethanol, followed by elution with ethyl acetate is also an effective and reproducible method for endocannabinoid extraction from biological fluids (Hill *et al*, 2008c). A modification of this approach has been described that utilizes C18-containing pipet tips to reduce the solvent and time needed for extraction (Sergi *et al*, 2013).

Quantification of the deuterated and endogenous endocannabinoids in each sample is usually accomplished with high-pressure liquid chromatography (HPLC) utilizing a C18 column, followed by mass spectrometry. With the availability of quadrupole, tandem mass spectrometers, species identification and precision of measurement are enhanced because the abundance of daughter ions rather than parent ions are measured. A recent method described the use of on-line sample trapping and microflow liquid chromatography coupled to the use of a tandem mass spectrometer that allowed for detection of the endocannabinoids in 50 μl of serum with the use of much less solvent than conventional methods (Kirkwood *et al*, 2016).

Serum AEA concentrations tend to fall between 1 and 5 nM while serum 2-AG concentrations range from 10–500 nM (see Table 2 in Hillard *et al* (2012)). Some of the likely reasons for the range in endocannabinoids in healthy individuals are discussed in the sections below, and include time of day and whether the individual has exercised or eaten recently. AEA can bind to the plasma protein, albumin (Giuffrida *et al*, 2000), although the equilibrium dissociation constant of bovine serum albumin for AEA is 55 nM at 37 °C (Bojesen and Hansen, 2003), suggesting that the majority of AEA in the circulation is not protein bound. Data regarding the binding of 2-AG to plasma proteins are not available; however, it is likely that its similarity to AEA and arachidonic acid would render it a low affinity ligand for albumin binding sites as well.

Endocannabinoids have been quantified in other biological samples obtained from humans, including saliva (Matias *et al*, 2012), hair (Krumbholz *et al*, 2013), semen (Amoako *et al*, 2010), breast milk (Lam *et al*, 2010), and amniotic fluid (Lam *et al*, 2010). Data from endocannabinoid measurements in these biological samples are far less frequent in the literature than measurements in the circulation, however, they could have important advantages. For example, measurement in the hair could provide a retrospective picture of endocannabinoids and the saliva is easier to obtain than blood, particularly in a non-hospital setting.

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Circulating endocannabinoids and metabolic regulation

The endocannabinoids play a very important role in the regulation of metabolism at systems and cellular levels. As was well stated in a recent review (Mazier *et al*, 2015): ‘The endocannabinoid system exerts regulatory control essentially on every aspect related to search, intake, metabolism and storage of calories…’. Activation of CB1R signaling by the endocannabinoids results in increased consumption of food. In the hypothalamus, tonic endocannabinoid-CB1R signaling functions to maintain food consumption and is suppressed by the homeostatic appetite regulator, leptin (Di Marzo *et al*, 2001). CB1 receptors are also present in hypothalamic nuclei that modulate energy homeostasis via interactions with ghrelin (Kola *et al*, 2008). In addition, activation of CB1R signaling enhances reward circuits through effects in the mesolimbic system (De Luca *et al*, 2012; Mahler *et al*, 2007; Melis *et al*, 2007), which potentiates hedonic (reward-driven) consumption of highly palatable foods in particular. Functional imaging of humans performing a reward-driven task following THC find significant attenuation of feedback to the reward circuits compared to placebo administration (van Hell *et al*, 2012). These studies suggest a particular role for CB1R signaling in feedback inhibition of reward seeking.

CB1R are also present in peripheral organs involved in energy metabolism, including adipose tissue, the endocrine pancreas, muscle and liver (Bermudez-Silva *et al*, 2012; Cota *et al*, 2006; Silvestri and Di Marzo, 2013). Increased CB1R signaling increases energy storage via stimulation of fat mass expansion, glucose uptake and adipogenesis in adipocytes (Vettor and Pagano, 2009); induction of lipogenesis in the liver (Osei-Hyiaman *et al*, 2005); and increased insulin secretion from the pancreas (Juan-Pico *et al*, 2006). CB1R signaling also increases processes that reduce energy utilization, including decreased brown fat thermogenesis (Verty *et al*, 2009) and glucose uptake into skeletal muscle (Liu *et al*, 2005). Thus, CB1R signaling is coordinated throughout the body to maximize intake and storage of energy. Clearly, this function of the endocannabinoid-CB1R system has great implications for obesity and other components of the metabolic syndrome in humans. In fact, a CB1R antagonist, rimonabant, was found to have significant, beneficial effects in humans to reduce body weight, improve lipid profiles and increase insulin sensitivity before it was removed from clinical use (Van Gaal *et al*, 2005). In addition, a recent study identified expression of the CB1R in the subfornical organ of the hypothalamus, which is increasing being recognized as a brain region that is responsive to changes in circulating signals of metabolic status (Hindmarch and Ferguson, 2016). This finding suggests that endocannabinoids in the circulation could contribute to the information that is utilized by the brain to alter behavior and physiologic responses.

Circulating Endocannabinoids, Food Consumption and Obesity

The data discussed above suggest the hypothesis that circulating endocannabinoids coordinate CB1R roles in energy intake and storage among various tissues and organs. In this section, data regarding relationships between circulating endocannabinoids and food consumption, and the

correlations among circulating endocannabinoids, body weight and measures of adiposity will be considered. Altogether, these findings support the hypotheses that endocannabinoid concentrations in the circulation are sensitive to several aspects of food consumption and dysregulated in obese and anorexic individuals.

The pattern of change in circulating AEA concentrations surrounding food consumption is consistent with its role as an orexigenic factor in homeostatic (hunger-driven) feeding. Presentation of food (prior to consumption) results in a significant increase in circulating AEA in normal weight (NW) and obese individuals (Gatta-Cherifi *et al*, 2012) followed by a small but reproducible fall in circulating concentrations of AEA over several hours following food consumption (Gatta-Cherifi *et al*, 2012; Monteleone *et al*, 2012, 2015). In NW individuals, the post-meal reduction in circulating AEA is independent of the hedonic value of the meal (Monteleone *et al*, 2012). Intravenous glucose administration also reduces circulating AEA for several hours following infusion in NW individuals (Di Marzo *et al*, 2009). Oral consumption of 250 ml of grape juice (41 g sugar) significantly elevated blood glucose levels and reduced circulating 2-AG but did not affect AEA concentrations (Feuerecker *et al*, 2012a), suggesting that there is a dose dependence to effects of sugars on endocannabinoids in the circulation. The suppressive effects of food and glucose on circulating AEA concentrations could be mediated by increased insulin, since exogenous infusion of insulin also reduces circulating AEA concentrations in the same time frame (Di Marzo *et al*, 2009). In agreement with this hypothesis, the effect of glucose to reduce circulating AEA was lost in insulin insensitive individuals (Di Marzo *et al*, 2009); however, another study found that a hyperinsulin/euglycemic clamp decreased circulating AEA concentrations in both insulin sensitive and insulin resistant obese individuals (Abdulnour *et al*, 2014). *In vitro* data demonstrate that normal but not insulin-resistant adipocytes respond to insulin with significant reductions in intracellular concentrations of both AEA and 2-AG (D'Eon *et al*, 2008).

The response of 2-AG to food is affected by its perceived hedonic value, suggesting that 2-AG is involved in reward-driven (hedonic) feeding. When NW individuals are presented with a favorite compared to a non-favored food, circulating 2-AG concentrations are significantly higher at food presentation, and during and after its consumption (Monteleone *et al*, 2012, 2015). Sleep deprivation promotes increased hedonic eating, particularly in the later part of the day (Benedict *et al*, 2012; Nedeltcheva *et al*, 2009; St-Onge *et al*, 2011) and also increases circulating 2-AG concentrations at mid-day (Hanlon *et al*, 2016). These findings suggest that circulating 2-AG concentrations reflect activation of reward circuits in the brain and could be a mediator of hedonic eating.

A small study found that neither underweight nor weight-restored individuals with anorexia nervosa exhibited an increase in 2-AG in response to presentation or eating a favorite food (Monteleone *et al*, 2015). These data are consistent with hypotheses that dysregulated evaluation of rewarding stimuli occurs in anorexia (Keating *et al*, 2012) and that circulating 2-AG is related to perceived reward. On the other hand, circulating concentrations of AEA declined after favorite and non-favored food consumption in both subgroups of anorexic individuals, patterns that did not differ from NW individuals (Monteleone *et al*, 2015). These data are consistent with the notion that circulating AEA concentrations are involved in homeostatic and not hedonic feeding processes.

A recent study found that olfactory threshold discrimination identification capacity is significantly lower in obese women than NW and is inversely related to circulating 2-AG concentrations (Pastor *et al*, 2016). These findings are somewhat counterintuitive since preclinical studies suggest that CB1R signaling in the olfactory epithelium is associated with enhanced sensitivity to oderants (Breunig *et al*, 2010) and THC exposure can enhance the sensory aspects of food consumption in humans (Brisbois *et al*, 2011). However, it is possible that the human data reflect downregulation of olfactory CB1 receptor signaling in response to high 2-AG concentrations.

Regardless of the motivation for overeating or the type of food consumed, excessive food intake over a prolonged period results in obesity. Food-induced changes in circulating endocannabinoids have been characterized in obese individuals to examine the hypothesis that high endocannabinoid tone is associated with obesity. The consumption-induced reduction in circulating AEA concentrations seen in NW individuals is blunted in insulin insensitive, obese individuals and the magnitude of the decrease in AEA following eating is significantly, inversely correlated with waist circumference (Gatta-Cherifi *et al*, 2012). Given that these individuals were insulin insensitive in addition to being obese, it is possible that loss of insulin sensitivity underlies both differences in waist circumference and the lack of AEA decrease following food consumption. Interestingly, the pre-meal increase in AEA is intact in obese individuals, suggesting that the mechanisms underlying the pre-meal increase and post-meal decrease in AEA are different. A second study also found that obese individuals exhibit a loss in the decline in AEA following food consumption, but this change occurs when favorite but not non-favored foods are consumed (Monteleone *et al*, 2016).

Multiple studies (Abdulnour *et al*, 2014; Bluher *et al*, 2006; Cote *et al*, 2007; Engeli *et al*, 2005; Fernandez-Aranda *et al*, 2014; Pastor *et al*, 2016) (but not all (Di Marzo *et al*, 2009)) have found that concentrations of 2-AG in blood harvested in the morning after an overnight fast are significantly higher in men and women with body mass index (BMI) values in the obese range compared to NW individuals. Although obese individuals with subcutaneous fat distributions also have elevated 2-AG compared to NW, those with a visceral fat distribution are higher still (Bluher *et al*, 2006). Furthermore, circulating concentrations of 2-AG are significantly, positively correlated with BMI, total body fat (particularly measures of visceral fat mass) (Bluher *et al*, 2006) and intra-abdominal adipose (IAA) tissue (Cote *et al*, 2007). One study also found a significant, positive correlation between BMI and circulating AEA concentrations in individuals with a range of BMI from 18 to 45 (Weis *et al*, 2010a). Studies that included only or mostly women found that AEA as well as 2-AG are significantly higher in obese than NW individuals (Engeli *et al*, 2005; Gatta-Cherifi *et al*, 2012). Conversely, a study in obese men with high amounts of IAA found that AEA concentrations in the morning are significantly lower than normal weight men or obese men with low IAA (Cote *et al*, 2007). It is possible that differences in the relative distributions of visceral and subcutaneous fat stores between men and women (Enzi *et al*, 1986) could underlie the sex differences observed in these studies. The one study that did not identify an increase in 2-AG in obese individuals studied a cohort without high visceral fat depots (Di Marzo *et al*, 2009), which further supports the visceral fat as the source of 2-AG in obesity. In one study (Abdulnour *et al*, 2014), insulin resistant obese had higher circulating 2-AG concentrations than insulin sensitive; however, the insulin resistant group also had a significantly higher BMI, which could underlie the higher circulating 2-AG. Taken together, these studies suggest that 2-AG enters the circulation from visceral, abdominal fat stores, so its concentration is highest in individuals with the highest amount of visceral fat. Given the roles of 2-AG to increase fat storage in adipose and liver (Mazier *et al*, 2015) and its ability to promote consumption of highly palatable foods through effects on the brain (Wei *et al*, 2016), these findings suggest that 2-AG-CB1 receptor signaling participates in a feed-forward cycle to promote further food consumption and increased body weight gain in the obese. One preclinical study has examined circulating endocannabinoid concentrations in mice made obese through long-term exposure to exogenous glucocorticoids and found that circulating AEA is nearly doubled in the treated, obese mice compared to controls (Bowles *et al*, 2015). 2-AG concentrations in the circulation were unchanged by the treatment, suggesting that obesity as a result of excess glucocorticoids is mediated by AEA rather than increased 2-AG.

Several studies have examined the expression of FAAH determined in adipose tissue biopsies in relationship to the circulating endocannabinoid concentrations. In postmenopausal women, adipose FAAH expression is reduced in obese women compared to normal weight; and is significantly, negatively correlated with circulating AEA (Engeli *et al*, 2005). Interestingly, the same study and another that included both men and women (Bluher *et al*, 2006) found that adipose FAAH expression is also significantly, negatively correlated with morning 2-AG concentrations. Although 2-AG can serve as a substrate for FAAH *in vitro* (Goparaju *et al*, 1998), preclinical studies in mice without FAAH expression show no changes in 2-AG content (Patel *et al*, 2005) which has lead to the widely held assumption that FAAH does not catabolize 2-AG *in vivo*. Indeed, the correlation between adipose FAAH expression and circulating AEA is stronger than the correlation with circulating 2-AG (Engeli *et al*, 2005). It is possible that reduced FAAH expression is a causative factor, resulting in elevated AEA, which, in turn primes the system to increase caloric intake and fat storage through CB1 receptor activation. Adipose fat stores are increased, resulting in an increase in circulating 2-AG, leading to the feed-forward cycle described above. Another study did not find a significant relationship between adipose FAAH activity and BMI or any other measure of metabolic disorder, instead, found that BMI was significantly, positively correlated with adipose MAGL activity in obese without diabetes (Cable *et al*, 2014). The authors of this study concluded that the rates of catabolism of AEA and 2-AG in adipose do not contribute to their circulating concentrations.

Preclinical findings suggest that a source of circulating endocannabinoids is the small intestine (Dipatrizio *et al*, 2011). In particular, sham feeding of rats with a lipid meal results in increased endocannabinoid contents in the jejunum via a signal carried by the vagus nerve. Lipid feeding reduced the catabolism of both AEA and 2-AG, resulting in increased tissue contents. Although not studied, it is possible that these lipids enter the portal system and contribute to elevations in circulating concentrations. A recent paper reported that a peripherally restricted CB1R antagonist reduced hyperphagia in mice fed a Western diet (ie, high in carbohydrate and fat), which supports a role for peripheral CB1R signaling in hyperphagia (Argueta and DiPatrizio, 2017).

Although it is not an endocannabinoid *per se*, preclinical studies suggest the hypothesis that the NAE, OEA, also has important effects on satiety and feeding behavior. In particular, administration of OEA to mice reduces food intake and body weight gain, likely through PPAR-alpha in the GI tract (Fu *et al*, 2003) and or through actions in the area postrema (Romano *et al*, 2017). OEA is synthesized by enterocytes of the small intestine in response to fat intake (Fu *et al*, 2007). In accord with the preclinical findings, hypercholesterolemic patients treated with diets enriched in oleic acid exhibited significantly higher concentrations of plasma OEA concentrations and a significant, negative relationship between plasma OEA and percent body fat (Jones *et al*, 2014). These data, together with the finding that circulating OEA and AEA concentrations do not change in parallel in response to eating (Monteleone *et al*, 2016), support the hypothesis that circulating OEA concentrations are regulated by a mechanism that differs from that regulating concentrations of AEA.

Relationships of Endocannabinoid Concentrations with Time of Day and Sleep

Normalized serum 2-AG concentrations exhibit a significant circadian rhythm in humans under conditions of standardized sleep timing and caloric intake (Hanlon *et al*, 2015). The average low point (nadir) of 2-AG concentration occurred in this study at 0400 hours, 5 h after going to bed and 9 h after the last food consumption. 2-AG concentrations rose steadily from that point, followed by an extended period of maximal concentrations between 1200 and 1500 hours, a time period that encompassed the midday meal at 1400 hours. The return to lower, nocturnal 2-AG concentrations began in the late afternoon/early evening, with a small plateau in 2-AG concentrations occurring between 1700 and 2000 hours, which surrounded the evening meal at 1900 hours. A remarkable threefold change in circulating 2-AG concentration occurred between the nadir and acrophase.

A second study from the same group found that reduction of the number of hours of sleep to four, while maintaining the same sleep period mid-point, produced a significant increase in the amplitude and peak concentrations of 2-AG (Hanlon *et al*, 2016). In addition, the time of the acrophase was shifted significantly from 1230 to 1430 hours and the mean 2-AG concentrations between 1400 and 2100 hours trended ($p<0.06$) higher. Thus, restricted sleep increases and extends the peak of 2-AG concentrations in the afternoon following sleep restriction. Interestingly, sleep restriction did not affect patterns of 2-AG in the morning and did not affect the nadir concentrations or timing. Instead, the effect of restricted sleep on 2-AG is delayed until later in the day.

In another study, 2-AG concentrations were also found to be elevated in healthy controls exposed to 4 days of restricted sleep, although the timing was different (Cedernaes *et al*, 2016). In that study, sleep was restricted to 4 h, but the period corresponded to the second half of the normal sleep period (ie, 0245–0700 hours). Under these conditions, 2-AG concentrations in the plasma are significantly elevated at 0830 following restricted sleep compared to normal sleep and are not different at 2030 hours. Midday 2-AG concentrations between normal and restricted sleep phases were not determined in this study. It is possible that the differences in timing of sleep in the restriction paradigm between this study and that by Hanlon (where the sleep midpoint was kept constant) (Hanlon *et al*, 2016) contributed to the difference in the time at which 2-AG was elevated. If this is the case, it suggests that sleep timing as well as number of hours regulate the chronicity of circulating 2-AG. In this regard, advancing the sleep schedule without changing the light-dark cycle has a moderate effect on the rhythms of melatonin and core body temperature (Danilenko *et al*, 2003), suggesting that sleep timing can function as a modulator of circadian rhythm. In any case, both studies identified increased circulating 2-AG following restricted sleep.

In a very small pilot study (five subjects) looking at three time points, AEA concentrations in the serum were found to be higher upon wakening (0730 hours) than late evening (2230 hours) (Vaughn *et al*, 2010). Furthermore, one night of complete sleep deprivation results in elevation of serum AEA concentrations later in the day. Both of these results suggest that AEA might have rhythms and relationships to sleep loss similar to those of 2-AG; however, more complete studies need to be carried out to confirm this conclusion.

These data place circulating endocannabinoids, particularly 2-AG, in the pantheon of molecules which alter metabolism and are circadian. In fact, 2-AG fills a unique role because it is an orexigenic mediator that exhibits its highest concentrations in midday and lowest during sleep. Neither the hormones controlled by the HPA axis nor adipokines and gut hormones reach peak concentrations in the middle of the normal feeding period (Copinschi *et al*, 2010).

As was pointed out in a commentary (Scheer, 2016) that accompanied (Hanlon *et al*, 2016), the increases in 2-AG and 2-OG began in the middle of the sleep episode, thus preceding breakfast, awakening and morning light exposure. The timing of food intake, transition from sleep to wakening, and light/dark cycles are all important circadian regulators, but the increases in circulating 2-acylglycerols precede these events, so are not likely driven by them. Glucocorticoids also rise in the morning, and data obtained in rodents indicate that glucocorticoids can rapidly increase 2-AG synthesis in the hypothalamus (Hill *et al*, 2010). While morning changes in 2-AG paralleled the rise in cortisol, the nadir and acrophase for cortisol were 3–4 h earlier than for 2-AG (Hanlon *et al*, 2015). Thus, 2-AG concentrations appear to have an endogenous, circadian rhythm that differs significantly from those of other metabolic regulators.

Activation of CB1R signaling provides a highly efficacious orexigenic signal (Cota *et al*, 2006) and the timing of the peak in circulating 2-AG has been hypothesized to engage CB1Rs throughout the body to promote eating during the middle of the active period (Hanlon *et al*, 2015). In light of the argument presented above that circulating 2-AG is involved in hedonic rather than homeostatic eating, a more specific hypothesis is that midday 2-AG rise promotes food intake for storage, rather than for immediate use. It is interesting in this regard that insulin sensitivity exhibits a robust decrease from morning to evening in healthy human volunteers (Saad *et al*, 2012; Van Cauter *et al*, 1997) which is consistent with increased 2-AG-mediated CB1R signaling over that time period. Thus, elevation of 2-AG *in situations* that could have a significant effect on future metabolic needs, such as chronic loss of sleep, could protect against starvation by promoting an increase in fat stores. This hypothesis is supported by the findings that sleep deprivation both increased feelings of hunger and drive to eat high-calorie foods in the afternoon and increased and shifted the 2-AG acrophase to later times (Hanlon *et al*, 2016).

The mechanisms that underlie the circadian changes in 2-AG and the shift in pattern that occurs with sleep deprivation are not known. However, given the fact that sleep deprivation is a significant stressor, it is possible that 2-AG concentrations are sustained as a result of stress-induced glucocorticoid elevation.

Circulating Endocannabinoids and Exercise

The hypothesis that circulating endocannabinoids coordinate a system-wide response to seek, consume and store energy leads logically to the hypothesis that increased energy utilization results in increased circulating endocannabinoids to replenish energy stores. In support of this hypothesis, multiple studies demonstrate that 30–90 min of moderate exercise increases circulating concentrations of AEA (Heyman *et al*, 2012b; Raichlen *et al*, 2012, 2013; Sparling *et al*, 2003) and other NAEs (Heyman *et al*, 2012b) immediately after the exercise period, although a recent study did not reveal an immediate increase in AEA (Cedernaes *et al*, 2016). AEA increases reached significance when heart rate was increased to about 75% of maximal (Raichlen *et al*, 2013); neither lower (ie, walking) nor near-maximal intensities of exercise affected AEA concentrations (Raichlen *et al*, 2012, 2013) suggesting a 'U-shaped' relationship between exercise and AEA mobilization. Well-trained healthy volunteers who participated in a 'strenuous' hike at altitude below 2100 meters and those who hiked and ascended approximately 2000 meters in altitude both exhibited significant increases in circulating AEA concentrations following the exercise period (Feuerecker *et al*, 2012b). Interestingly, hypoxic stress of exercise at high altitude appeared to potentiate the increase in AEA in this study. A preclinical study utilizing hepatic ischemia-reperfusion injury also found that 2-AG is

increased in response to ischemia, which could reflect regulation of endocannabinoids by the state of oxygenation of tissues (Kurabayashi *et al*, 2005).

A few studies have examined chronic effects of physical exercise on baseline endocannabinoids. A study of NW and obese women found that moderate-vigorous physical activity measured over 6 days is positively correlated with circulating AEA concentrations (Fernandez-Aranda *et al*, 2014). Interestingly, high circulating AEA concentrations are also associated with high BMI. On the other hand, no differences in basal concentrations of AEA and 2-AG were found between active and sedentary NW men in another study (Gasperi *et al*, 2014).

The effects of exercise on circulating 2-AG concentrations have been less consistent. Studies that employed 30–90 min of sustained exercise and observed increases in AEA did not find an increase in 2-AG (Heyman *et al*, 2012b; Raichlen *et al*, 2012, 2013; Sparling *et al*, 2003). However, another study employing an exercise regimen known to induce exercise-induced hypoalgesia (3 min of isometric contractions of the forearm) found that 2-AG is increased very significantly, in addition to the NAEs (Koltyn *et al*, 2014). Another study found an increase in 2-AG measured in blood collected 15 min after a 30 min exercise period (Cedernaes *et al*, 2016); however, the subjects in this study were not given food in the morning of the experiment, which could have resulted in a more physiologically stressful paradigm than exercise alone. Thus, the available data are most consistent with a hypothesis in which moderate levels of sustained (ie, more than 30 min) exercise increase AEA and other NAEs in the circulation while 2-AG increases in response to short bursts of activity, perhaps specifically in response to isometric contractions.

It is possible that the increased endocannabinoids in the circulation following exercise come from skeletal muscle. Messenger RNAs encoding NAPE-specific phospholipase D (Hutchins-Wiese *et al*, 2012), and diacylglycerol lipase (Crespillo *et al*, 2011) are present in skeletal muscle. PEA and another NAE, stearoylethanolamide, are increased by exercise in microdialysates of the trapezius muscle in individuals with chronic neck/shoulder pain (Ghafouri *et al*, 2013), providing additional support for the hypothesis that endocannabinoids can be produced in muscle. It is well established that exercise increases circulating glucocorticoid concentrations (Urhausen *et al*, 1995); and exogenous glucocorticoid treatment produces a rapid increase in brain AEA concentrations (Hill *et al*, 2010), suggesting a possible link between glucocorticoid receptor activation and AEA synthesis.

Endocannabinoid mobilization during exercise is very consistent with the role of CB1R signaling in the regulation of metabolism discussed above. Activation of CB1R in the liver and adipose move the system toward energy storage and thus can serve to both restrain energy use and contribute to recovery of energy stores following exercise (Pagotto *et al*, 2006). DiMarzo and colleagues have suggested an interesting hypothesis that AEA and other NAEs could produce positive metabolic effects locally in skeletal muscle, including enhanced glucose uptake, improved insulin action, and mitochondrial biogenesis via activation of PPARγ (Heyman *et al*, 2012a). Although endocannabinoids can also act centrally to increase food consumption, a recent study found that exercise-induced increases in endocannabinoids were not correlated with feelings of subjective hunger (Cedernaes *et al*, 2016), suggesting that any metabolic role for the circulating endocannabinoids is likely to be more peripheral than central. A study in healthy cyclists demonstrated a significant, positive correlation between the increase in circulating AEA and an increase in the neurotrophic factor, BDNF, following a 90 min exercise period (Heyman *et al*, 2012b). Although the directionality of this relationship is not known, acute administration THC to healthy, non-cannabis-using humans also increases serum BDNF concentrations (D'Souza *et al*, 2009), suggesting that BDNF synthesis/release is evoked by CB1R activation. Preclinical studies in the CNS are also consistent with CB1R regulation of BDNF (Khaspekov *et al*, 2004; Marsicano *et al*, 2003). These data suggest that endocannabinoids could be the mediators of exercise-induced increases in BDNF (Szuhany *et al*, 2015) and, thus, of the hypothesized effects of exercise on cognitive function and mood (Phillips *et al*, 2014).

Go to:

Circulating endocannabinoids and inflammation

Circulating endocannabinoids are significantly increased in several conditions in which there is systemic inflammation. For example, both 2-AG and AEA are folds-higher in the serum of patients with endotoxic shock than control serum (Wang *et al*, 2001). Circulating endocannabinoids are increased in patients with cirrhosis (Fernandez-Rodriguez *et al*, 2004); chronic hepatitis C infection (Patsenker *et al*, 2015); exposure to solar ultraviolet radiation (Felton *et al*, 2017); congestive heart failure (Weis *et al*, 2010b); and atherosclerosis (Maeda *et al*, 2009). Similarly, rats with acute pancreatitis also exhibit significantly elevated circulating AEA concentrations, particularly in animals with a severe, necrotizing pancreatitis (Matsuda *et al*, 2005). When activated, macrophages (Di Marzo *et al*, 1999; Varga *et al*, 1998), platelets (Varga *et al*, 1998), T and B cells (Sido *et al*, 2016) release endocannabinoids *ex vivo*, and so could be a source of circulating endocannabinoids. On the other hand, a recent study carried out in patients undergoing hematopoietic stem cell transplantation (HCT) suggests that bone marrow-derived immune cells *per se* may not be an important source of 2-AG (Knight *et al*, 2015). In particular, no change in 2-AG concentrations occurred between the period in which the HCT patient's immune system is ablated and after immune system reconstitution. Preclinical studies suggest two alternative hypotheses. First, it is possible that non-immune cells respond to inflammatory mediators with increased endocannabinoid release as has been shown in inflamed hepatocytes (Batkai *et al*, 2007). Second, inflammation results in a reduction in 2-AG catabolism by splenic carboxylesterase 2 g in the spleen (Szafran *et al*, 2015), suggesting that the rise in 2-AG during inflammation could be the result of spillover from the spleen, which is enhanced as a result of decreased 2-AG hydrolysis in that tissue.

2-AG is an agonist of the CB2 receptor (Soethoudt *et al*, 2017), a GPCR that is expressed constitutively by human B and T cells; macrophages and, to a lesser extent, neutrophils (Galiegue *et al*, 1995). CB2R activation is associated with a reduction in proinflammatory cytokine release (Leleu-Chavain *et al*, 2013; Patsenker *et al*, 2015) and with reduced migration of T cells into the brain (Maresz *et al*, 2007). The findings that 2-AG can be elevated during inflammation and that it activates an anti-inflammatory receptor suggest the hypothesis that endocannabinoids function as a feedback system to dampen inflammation. A recent preclinical study highlights this relationship. In this study, antigen activation of macrophages and dendritic cells resulted in increased 2-AG and CB2R antagonist treatment increased antigen-specific immune responses in mice, suggesting that 2-AG/CB2R activation suppresses innate and adaptive immune responses in mice (Dotsey *et al*, 2017).

In contrast to these hypotheses, several studies have found significant, positive correlations between circulating 2-AG concentrations and concentrations of the pro-inflammatory cytokine interleukin-6 (IL-6) (Knight *et al*, 2015; Weis *et al*, 2010a), data that do not reconcile with this hypothesis. If 2-AG is anti-inflammatory even to a small extent, one would expect a negative relationship between IL-6 and 2-AG. In this regard, 2-AG potentiates LPS-stimulated IL-6 expression in a macrophage cell line (Marazzi *et al*, 2011). On the other hand, plasma concentrations of 2-AG and the cytokine, tissue necrosis factor alpha, were found to be significantly negatively correlated in another study (Koppel *et al*, 2009).

The relationships between 2-AG and cytokines in the circulation need further study as does the fundamental question of whether circulating endocannabinoids participate in regulation of inflammation in humans. In this regard, several of the conditions described above in which circulating endocannabinoids are elevated also result in inflammation, including acute bouts of exercise (Ruderman *et al*, 2006) and obesity (Marsland *et al*, 2010), data that support the possibility that there are connections between endocannabinoids and inflammation. Activation of CB1R signaling in the context of inflammation can be a contributing factor to fibrosis and other detrimental effects of chronic inflammation (Iyer *et al*, 2017). Circulating concentrations of AEA are elevated in patients with cirrhosis and their concentrations are positively correlated with negative indices of liver function (Caraceni *et al*, 2010).

Go to:

Circulating endocannabinoids and stress

Exposure to physical and psychological stressors result in coordinated behavioral, endocrine and neuronal changes that increase chances for survival if an injury does happen. Acute stress responses include activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Stress exposure also produces consistent and reproducible increases in inflammation systemically and in the brain, likely as a downstream

consequence of sympathetic activation (Dantzer *et al*, 2008). For example, acute psychological stress results in an immediate increase in natural killer T cells and interleukin 1beta (IL-1β) (Yamakawa *et al*, 2009) and a delayed (90 min) increase in IL-6 (Steptoe *et al*, 2007). Sympathetic activation results in migration of monocytes and macrophages into the circulation and triggers release of IL-1ß and other cytokines (Yamakawa *et al*, 2009).

Preclinical data support the hypotheses that endocannabinoid signaling is altered by stress and mediates or modulates stress responses (Hillard, 2014). Evidence obtained in brain demonstrate that 2-AG mobilization can occur downstream of glucocorticoid receptor activation (Di *et al*, 2005) while AEA is elevated by corticotropin releasing hormone receptor activation, secondary to inhibition of FAAH (Gray *et al*, 2015). Endocannabinoid/CB1R signaling inhibits the activation of the HPA axis by stress (Hill *et al*, 2009a) and enhances recovery following stress offset (Hill *et al*, 2011).

CB1R are present on sympathetic nerve terminals and their activation results in sympathomimetic effects in preclinical studies (Ishac *et al*, 1996; Pfitzer *et al*, 2005). However, AEA infused intra-arterially in the human forearm failed to affect blood flow (Movahed *et al*, 2005), suggesting that CB1R activation may not affect release from sympathetic nerve terminals in healthy humans. Preclinical studies demonstrate that CB1R signaling in the nucleus tractus solitarius (NTS) potentiates the sympathoinhibitory responses to activation of the baroreceptor reflex (Chen *et al*, 2010; Seagard *et al*, 2004, 2005). On the other hand, CB1R activation in the periaqueductal gray (PAG) increases activity of the SNS (Dean, 2011).

Effects of Acute Stress on Circulating Endocannabinoids

Activation of the SNS as a result of inducing postural hypotension with a head up tilt is accompanied by a significant increase in 2-AG concentrations (Schroeder *et al*, 2009). However, associations between 2-AG concentrations and measures of sympathetic activation were not found (Schroeder *et al*, 2009). In the same study, circulating concentrations of AEA were significantly, positively correlated with the ability of patients to tolerate the procedure without fainting (Schroeder *et al*, 2009). This result suggests a positive relationship between AEA and SNS activity.

Vestibular activation of the SNS triggers nausea and vomiting (Balaban, 1999); and parabolic flight maneuvers that trigger motion sickness produce an immediate increase in circulating AEA concentrations and a delayed (recovery phase) increase in 2-AG (Chouker *et al*, 2010). Increases in both 2-AG and AEA concentrations are associated with less motion sickness, which is consistent with preclinical and human data that exogenous activation of CB1R signaling reduces nausea and vomiting (Sticht *et al*, 2015).

Hyperemesis is a significant physiological stress and is accompanied by increases in both SNS and HPA axis activation. Cyclic vomiting syndrome (CVS) is a functional disorder characterized by relapsing and remitting bouts of prolonged nausea and vomiting. Since endocannabinoids are elevated during stress and can reduce nausea and vomiting, circulating endocannabinoids were compared between sick (active vomiting) and well phases in individuals with CVS (Venkatesan *et al*, 2016). Concentrations of both AEA and 2-AG are elevated in the sick compared to well phases, but the increases did not reach statistical significance (p=0.09 for both). Concentrations of both PEA and OEA were significantly increased and positively correlated with the amount of distress. No significant associations between circulating endocannabinoid concentrations and circulating cortisol or salivary amylase measured at the same time were found in this study.

Acute exposure to psychological stress also elevates circulating endocannabinoid concentrations. A commonly used procedure to evoke psychological stress in humans in a laboratory setting is the Trier Social Stress Test (TSST). There are various forms of the TSST, but most include public speaking and solving difficult arithmetic problems (Allen *et al*, 2014). The TSST results in activation of both the SNS and HPA axis as well as an increase in perceived anxiety. Administration of a seventeen-minute version of the TSST resulted in a significant increase in 2-AG concentrations immediately afterwards that returned to basal concentrations 30 min after the end of the stress (Hill *et al*, 2009b). Serum AEA concentrations were not significantly different from baseline at either time point. However, a second study found that a shorter stress period (5 min) produced no significant change in 2-AG concentrations, but significantly increased circulating AEA concentrations immediately after the stress (Dlugos *et al*, 2012). Interestingly, this study also found preliminary evidence of differences in the endocannabinoid response among males and females in each phase of their menstrual cycles, with males exhibiting a greater increase from baseline than females, particularly females in the follicular phase. This study also found preliminary evidence of differences in the endocannabinoid response to stress between those of African and Caucasian descent. The change in PEA from baseline to after stress was significantly, positively correlated with the change in cortisol measured in the same samples; the change in AEA concentration was not significantly associated with cortisol.

A study in which imagery of a previous stressful experience was administered found that AEA concentrations were not changed from baseline but were significantly lower than baseline 75 min after the end of the stress (Mangieri *et al*, 2009), which is reminiscent of the effects of food consumption on circulating AEA concentrations discussed above.

Thus, while these studies all demonstrate that circulating endocannabinoid concentrations are sensitive to acute, psychological stress, they also indicate that the relationships are complex, time-dependent, and affected by sex. Circulating concentrations of both AEA and 2-AG can be affected by acute stress, but studies of associations with the primary stress effector systems, the HPA axis and SNS, do not consistently find evidence of a direct relationship between activation of these systems and concentrations of endocannabinoids in the circulation. However, preclinical studies indicate that the function of elevated endocannabinoids during stress is to buffer the stress response and hasten return to homeostasis through inhibition of the HPA stress response in particular. Thus, if endocannabinoids in the circulation are both downstream and upstream of the HPA axis, the relationships between endocannabinoid and cortisol concentrations would likely be nonlinear and dependent upon timing and individual responsivity of both systems. It is also possible that changes in endocannabinoid concentrations occur with a time delay, so that concentrations of cortisol or measures of SNS are not related when examined at the same point in time. Models that allow for these complexities to be assessed are needed to answer this question.

Circulating Endocannabinoids and Chronic SNS or HPA Axis Activation

Although there is little evidence that circulating endocannabinoids are associated with any measure of SNS activation at baseline, several studies suggest that AEA is associated with SNS-mediated physiological effects in various pathological conditions. For example, AEA concentrations are higher in individuals with sleep apnea and are strongly, positively correlated with both systolic and diastolic blood pressure, independent of BMI and insulin resistance (Engeli *et al*, 2012). Interestingly, depressed women also exhibit a significant, positive correlation of circulating AEA concentrations and diastolic blood pressure, with a trend (p<0.07) toward a significant association with systolic pressure as well (Ho *et al*, 2012). Intravenous injections of AEA in experimental animals reduce blood pressure (Ho and Gardiner, 2009), suggesting that the relationship underlying the correlation between circulating AEA concentrations and blood pressure is that elevated blood pressure results in increased AEA. Indeed, increased blood pressure in rats produces an elevation of AEA concentrations in the NTS (Seagard *et al*, 2004). On the other hand, circulating concentrations of AEA are significantly reduced in individuals with preeclampsia, a condition associated with hypertension in late pregnancy (Molvarec *et al*, 2015), suggesting that increased blood pressure alone is not sufficient to elevate circulating AEA concentrations.

An interesting recent study examined circulating endocannabinoid concentrations in individuals who were isolated from family and friends for more than 500 days in a study simulating a trip to Mars (Yi *et al*, 2016). Individuals in this condition exhibited significant reductions in positive emotions and increased SNS activation. Circulating concentrations of 2-AG but not AEA were significantly reduced at the end of this significant, chronic stress. 2-AG concentrations were significantly, positively correlated with EEG alpha activity (which was suppressed by the isolation) and significantly, negatively correlated with circulating cortisol. These very interesting findings suggest that 2-AG is brain-derived and driven by excitatory signaling, which is consistent with considerable data that glutamate increases 2-AG synthesis (Hillard, 2015). These findings are also consistent with the role of 2-AG to reduce HPA axis activation (Hillard *et al*, 2016). It is interesting that circulating 2-AG concentrations and measures of positive emotion were not associated in this study, given the evidence below that 2-AG has been found in other studies to be negatively associated with symptoms of depression.

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Circulating endocannabinoids and reward, anxiety and cognition

As has been discussed in preceding sections, CB1R signaling is involved in regulation of mood; modulates reward seeking behavior and hedonia; and is involved in behavioral responses to threat and stress. Some of the earliest studies in which circulating endocannabinoids were measured examined their relationships to indices of mood, anxiety and perception. These studies have the underlying assumption that circulating concentrations are affected by synaptic endocannabinoid tone, particularly in limbic brain regions. However, the only preclinical study that has tested this directly disputes this assumption (Hill *et al*, 2008a). In this study, rats exposed to chronic, unpredictable stress exposure had reduced endocannabinoid concentrations throughout the limbic regions of the brain, while plasma concentrations of AEA and 2-AG were significantly increased. However, the data that have come from human studies have largely found correlations between circulating endocannabinoids and measures of mood and anxiety in particular, suggesting that CNS function is somehow translated into alterations in circulating endocannabinoids.

Endocannabinoids and Anxiety

Several studies have found that circulating AEA concentrations are inversely related to both cognitive and somatic measures of anxiety (Dlugos *et al*, 2012; Hill *et al*, 2008c). In accordance with these data, carriers of FAAH C385A have significantly increased circulating AEA concentrations, and anxiety evoked by guided imagery of their trauma decayed more quickly than those homozygous for C at this position (Spagnolo *et al*, 2016). However, circulating endocannabinoids were not altered by touch massage, a procedure that produced a significant reduction in feelings of anxiety, although concentrations of PEA were significantly increased by this procedure (Lindgren *et al*, 2015).

Circulating endocannabinoids are altered in individuals diagnosed with post-traumatic stress disorder (PTSD). In particular, circulating concentrations of 2-AG are significantly lower in individuals with PTSD that occurred as a result of the World Trade Center attacks (Hill *et al*, 2013). The 2-AG decrease remained significant after correction for sex, alcohol use, and depression. Significantly lower circulating 2-AG concentrations without a difference in AEA were also found in a study of individuals with PTSD as a result of childhood sexual abuse compared to controls (Schaefer *et al*, 2014). On the other hand, hair OEA concentrations were significantly reduced in war survivors suffering from PTSD compared to survivors without the diagnosis and strongly, negatively correlated with symptom severity (Wilker *et al*, 2016). It is possible that measurement in the hair provides a more stable and reliable measurement, particularly for a chronic disorder, than a single time point measurement in the blood.

A few observations from these studies shed light on the possible relationships between circulating endocannabinoids and symptoms of PTSD. Although no group effects were identified, AEA concentrations were significantly, negatively associated with the number of intrusive symptoms in those with chronic PTSD (Hill *et al*, 2013). A study of alcoholics with PTSD found that the FAAH C385A SNP is associated with reduced hyperarousal (Spagnolo *et al*, 2016).

On the other hand, a study of war refugees with average scores of greater than 90 on the clinical administered PTSD scale (CAPS) and on average more than eight episodes of trauma exhibited significantly increased concentrations of both 2-AG and AEA compared to trauma-exposed individuals without PTSD and controls (Hauer *et al*, 2013). The contrast between these data and the other studies suggests that the amount of distress, not just the diagnosis of PTSD, could be an important determinant of endocannabinoid regulation.

Endocannabinoids, Reward and Depression

As was discussed above, increased circulating 2-AG concentrations are associated with enhanced hedonic food consumption, perhaps as a result of CB1R-mediated activation of reward seeking. Exercise has reported beneficial effects on mood, feelings of vigor, and anxiety, which are effects mediated by the CNS. Exercise-induced increases in AEA are significantly positively correlated with increased positive affect on the Positive and Negative Affect Schedule (PANAS) in one study (Raichlen *et al*, 2012), suggesting that recruitment of AEA-mediated signaling could contribute to the rewarding or reinforcing effects of exercise. Exercise-induced increases in AEA are positively correlated with circulating concentrations of BDNF, which is also associated with elevation of mood (Heyman *et al*, 2012b).

Several studies have examined the acute effects of drugs that are associated with increased pleasurable feelings on circulating endocannabinoid concentrations. Consumption of 250 ml of red wine with low sugar content resulted in reductions in AEA and 2-AG at 20 and 45 min after consumption, respectively (Feuerecker *et al*, 2012a). Healthy volunteers receiving an intravenous dose of 0.1 mg/kg THC responded with small increases in both 2-AG and AEA in the plasma at 30 min, followed by marked reductions in both 5 h after the THC administration (Thieme *et al*, 2014). The effects of THC are particularly interesting as they suggest that the endocannabinoid system is regulated by cannabinoid receptor ligand concentrations, so that endocannabinoid concentrations are reduced in the face of exogenous agonist consumption.

Guided imagery procedures that produce alcohol desire and craving in social drinkers increase circulating concentrations of AEA, which were positively correlated with alcohol craving and increased heart rate (Mangieri *et al*, 2009). Importantly, the relationship between AEA and alcohol craving is lost in recently abstinent alcoholics, suggesting that endocannabinoid signaling within the reward circuit is dysregulated during abstinence. Baseline concentrations of AEA in the circulation were found to be significantly lower in abstinent alcoholics than social drinkers as well.

Interestingly, circulating AEA concentrations are also significantly lower in runners who meet criteria for exercise dependence compared to runners who carry out similar amounts of activity but do not exhibit exercise dependence (Fergusson and Boden, 2008). Individuals who met criteria for exercise dependence exhibit both greater mood disturbance and significantly lower basal levels of AEA when deprived of exercise than runners who do not exhibit exercise dependence (Antunes *et al*, 2016). While these data suggest deficient endocannabinoid signaling could contribute to dysphoria during abstinence, resumption of exercise resulted in elevation of mood without an increase in circulating AEA, which does not support this hypothesis. A study that investigated a large number of NAEs and monacylglycerols (MAGs) in plasma from abstinent cocaine addicts found NAEs were increased while MAGs were decreased (Pavon *et al*, 2013). However, neither AEA nor 2-AG were affected specifically in this study.

Several studies have found that circulating concentrations of both 2-AG and AEA are significantly altered in women diagnosed with depression. 2-AG concentrations were significantly lower in those newly diagnosed with major depression compared to controls with a significant, negative relationship between circulating 2-AG and the duration of depressive symptoms (Hill *et al*, 2008c), which suggests the hypothesis that endocannabinoid deficiency contributes to depressive symptoms. Interestingly, AEA concentrations were increased in women diagnosed with minor depression, which the authors argued could be a compensatory mechanism to maintain elevated mood. In a second study of depressed women, baseline concentrations of both AEA and 2-AG were significantly lower than in controls (Hill *et al*, 2009b). Interestingly, 2-AG mobilization in response to the TSST was not different between control and depressed women (Hill *et al*, 2009b), suggesting that the mechanisms involved in the endocannabinoid response to acute stress and their role in depression are different. A study of individuals undergoing HCT found a significant, negative association between circulating 2-AG concentrations and depressive symptoms (Knight *et al*, 2015). On the other hand, a study of patients with osteoarthritis found that circulating 2-AG concentrations were significantly, positively associated with depression scores on the Hospital Anxiety and Depression Scale and negatively correlated with memory performance scores (La Porta *et al*, 2014). More work needs to be done to determine whether circulating endocannabinoid concentrations are related to specific depression symptoms or clusters of symptoms.

Endocannabinoids and cognition

Cannabis use in humans is associated with impaired executive function, including reduced behavioral flexibility (Ramaekers *et al*, 2006). A study in 150 healthy women showed a significant, negative correlation between 2-AG and cognitive flexibility performance assessed using the Wisconsin Card Sorting Test (WCST) (Fagundo *et al*, 2013), which is consistent with the hypothesis that excessive CB1R activation, likely in the prefrontal cortex, reduces executive functioning. On the other hand, the same study found a positive correlation between circulating AEA concentrations and behavioral flexibility in both the WCST and the Iowa Gambling Task.

The roles of cannabinoids and endocannabinoid signaling in memory formation, retrieval and extinction have been studied extensively in preclinical

models (Kruk-Slomka *et al*, 2016). While it is clear that memory is regulated by CB1R signaling, there is considerable variability in pharmacological studies that are likely related to dose, timing and differences in model systems. As noted above, circulating 2-AG concentrations are significantly, negatively correlated with memory in individuals with osteoporosis (La Porta *et al*, 2014). On the other hand, plasma 2-AG concentrations are significantly higher in individuals diagnosed with Alzheimer's Disease (AD) compared to matched controls and are positively correlated with memory and attention in those with AD (Altamura *et al*, 2015). Yet another study found no differences in circulating endocannabinoid concentrations between patients with AD and controls (Koppel *et al*, 2009). The general anesthetic, propofol, has efficacy as a FAAH inhibitor and elevates brain AEA concentrations in mice (Patel *et al*, 2003). In rats, propofol treatment after training in an inhibitory avoidance task significantly increased latencies 48 h later, which is consistent with stronger memory consolidation (Hauer *et al*, 2011). This effect of propofol was blocked by rimonabant, consistent with a CB1R-mediated enhancement of memory. However, humans tend to exhibit amnesia with propofol treatment (Pryor *et al*, 2015), and humans do not exhibit an increase in circulating AEA after propofol treatment (Jarzimski *et al*, 2012; Schelling *et al*, 2006) so whether this mechanism is operative remains to be determined.

Cognitive deficits are a core symptom of schizophrenia and the degree of cognitive deficiency is a primary factor in functional impairment (Green *et al*, 2000). There is evidence that chronic, heavy use of cannabis is associated with some of the cognitive deficits in schizophrenia (Solowij and Michie, 2007), which suggests the hypothesis that endocannabinoid signaling is dysregulated in schizophrenia. Indeed, concentrations of AEA in the cerebrospinal fluid (CSF) of first episode schizophrenics are very significantly higher than control (Leweke *et al*, 1999). But contrary to the hypothesis that dysregulated endocannabinoid signaling contributes to disease symptoms, CSF concentrations of AEA are negatively correlated with psychotic symptoms in unmedicated patients (Giuffrida *et al*, 2004). Similarly, individuals in the initial, prodromal state of psychosis with the lowest CSF AEA concentrations had the highest risk for converting to a full psychotic episode (Koethe *et al*, 2009). These findings suggest the hypothesis that elevation of CSF AEA reflects a beneficial recruitment of CNS AEA signaling. No differences in circulating AEA were detected between schizophrenics and controls in which robust differences in CSF concentrations were found (Leweke *et al*, 2007) and in another study (Desfosses *et al*, 2012). However, other studies have found increased circulating AEA concentrations in schizophrenics compared to controls (De Marchi *et al*, 2003; Potvin *et al*, 2008). Serum AEA concentrations were increased in response to treatment with cannabidiol, which also alleviated the symptoms of schizophrenia (Leweke *et al*, 2012). Circulating AEA and 2-AG concentrations are significantly higher in patients diagnosed with borderline personality disorder, a psychiatric disorder that has some of the positive symptoms of schizophrenia, than controls (Schaefer *et al*, 2014).

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Circulating endocannabinoids and pain

Preclinical and human studies support the overall hypothesis that CB1R and CB2R activation both result in reduced pain perception (Piomelli *et al*, 2014). In addition, data are emerging that PEA is anti-inflammatory and analgesic (Mattace Raso *et al*, 2014). On the other hand, there are mechanisms by which both 2-AG and AEA could increase pain sensation. 2-AG has been hypothesized to serve as a precursor for arachidonic acid (Kozak *et al*, 2000) and is converted by COX-2 to oxygenated products that increase pain perception (Hu *et al*, 2008); and AEA activates pro-nociceptive TRPV1 channels (Zygmunt *et al*, 1999). Thus, endocannabinoids have the potential to increase or decrease the perception of pain, depending upon the mechanism of action.

Increases in circulating AEA concentrations occur in patients with chronic pain syndromes, such as fibromyalgia (Kaufmann *et al*, 2008) and complex regional pain syndrome (Kaufmann *et al*, 2009) when compared to controls without pain. A relatively common single nucleotide polymorphism in the gene for FAAH that results in elevated circulating concentrations of AEA (rs324420) (Sipe *et al*, 2010) is associated with lower sensitivity to cold pain (Cajanus *et al*, 2016), suggesting that higher circulating AEA concentrations seen in chronic pain could reduce pain perception.

Increases in circulating 2-AG concentrations have also been reported in chronic pain, including neuromyelitis optica (NMO) (Pellkofer *et al*, 2013) and osteoarthritis (La Porta *et al*, 2015). In NMO, 2-AG is negatively correlated with mechanical pain thresholds, suggesting an analgesic role (Pellkofer *et al*, 2013). However, in osteoarthritis, 2-AG is positively correlated with knee pain, suggesting that it contributes to pain perception (La Porta *et al*, 2015).

In a dietary intervention study in chronic headache sufferers, increased omega-3 and reduced omega-6 fatty acid consumption was used to alter the circulating concentrations of AEA and 2-AG, which were both reduced by this intervention (Ramsden *et al*, 2015). Circulating concentrations of AEA and 2-AG were both significantly, positively correlated with the numbers of daily severe headaches, suggesting roles in the etiology of headache. Interestingly, this dietary intervention produces very significant increases in the ethanolamide and 2-acylglycerol of docosahexaenoic acid (DHA), both of which were inversely correlated with the number of severe daily headaches.

Exercise reduces the perception of pain, termed exercise-induced hypoalgesia (EIH), which occurs through both opioid and non-opioid mechanisms (Koltyn, 2000). In an early review, Dietrich and McDaniel speculated that endocannabinoids could be responsible for non-opioid EIH (Dietrich and McDaniel, 2004). In support of this hypothesis, Koltyn and colleagues demonstrated that brief periods of isometric exercise produce non-opioid-dependent EIH and a very significant increase in circulating 2-AG concentrations (Koltyn *et al*, 2014). Interestingly, correlational analyses did not support a significant relationship between circulating 2-AG (or AEA) concentrations and any measure of pain responsivity. However, CB1R are densely expressed on peripheral nerve terminals of A-delta and C primary afferents innervating skeletal muscle (Hohmann and Herkenham, 1999), so it is possible that the endocannabinoids increased by muscle activity act locally to produce EIH. This could explain the lack of correlation between endocannabinoids in the circulation and EIH.

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Summary and future studies

While our current state of understanding of the role of the endocannabinoid system in humans has been enhanced by measurement of circulating endocannabinoids, many of the studies have examined only a few individuals and only single time points. Recent studies showing that 2-AG is significantly circadian and that there are differences in the response to stress among men and women and, potentially, among races indicate that large, comprehensive studies that include a diverse population of individuals will provide the best data. Careful consideration of the time of sampling, and better yet, determination of endocannabinoids at multiple time points or after a stress or exercise challenge could provide important information that cannot be obtained at a single time point.

An important question that is particularly related to the studies of the relationships between circulating endocannabinoids and psychiatric disorders is whether circulating endocannabinoids are a biomarker for brain endocannabinoid signaling. Aside from the correlations and associations described above, the available data suggest that brain concentrations are not related to peripheral concentrations in a straightforward manner. For example, CSF AEA concentrations are increased multifold without consistent changes in their circulating concentrations, which indicates that overflow might not be consistent, particularly from the brain (Jumpertz *et al*, 2011). In addition, a preclinical study that examined both brain and peripheral endocannabinoids found no relationship between brain and plasma concentrations of 2-AG or AEA in chronically stressed rats (Hill *et al*, 2008b). It is possible that CNS function is translated into circulating endocannabinoids through changes in neuronal or endocrine outputs that drive alterations in endocannabinoid synthesis or degradation in peripheral tissues.

In my opinion, it is time for preclinical studies, where the relationships among circulating endocannabinoids and tissues can be determined, to catch up to the data obtained in humans. Preclinical models can help us to understand the extent to which circulating concentrations of endocannabinoids are related to brain concentrations; the degree to which circulating concentrations represent signaling pools; and, most importantly, whether or not circulating endocannabinoid concentrations can function as biomarkers of endocannabinoid signaling status. Currently, the answers to these

fundamental questions are unknown.

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A Role for the Endocannabinoid System in the Increased Motivation for Cocaine in Extended-Access Conditions

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Abstract

Extended access to cocaine produces an increase in cocaine self-administration in rats that mimics aspects of compulsive drug intake in human addicts. Although emerging evidence implicates the endogenous cannabinoid system in aspects of opioid and ethanol addiction, a role of the endocannabinoid system in cocaine addiction remains widely inconclusive. Here, we investigate the effects of systemic and intra-accumbal administration of the CB1 antagonist SR141716A (Rimonabant) on cocaine self-administration (0.5 mg/kg/infusion) under a progressive ratio (PR) schedule in rats with extended [long access (LgA); 6 h/d] or limited [short access (ShA); 1 h/d] access to cocaine. LgA rats, but not ShA rats, showed an increase in cocaine intake as previously reported, and responding for cocaine by LgA rats was higher than in ShA rats under a PR schedule. Systemic SR141716A induced a dramatic dose-dependent decrease in the breakpoint for cocaine by LgA rats, whereas only the highest dose of the antagonist had a significant effect in the ShA group. Anandamide levels in the nucleus accumbens (NAc) shell were decreased in ShA rats but unchanged in LgA rats during cocaine self-administration. Both phosphorylated and total CB1 receptor protein expression were upregulated in LgA rats in the NAc and the amygdala compared with ShA and drug-naive rats, 24 h after last cocaine session. Finally, intra-NAc infusions of SR141716A reduced cocaine breakpoints selectively in LgA animals. These results suggest that neuroadaptations in the endogenous cannabinoid system may be part of the neuroplasticity associated with the development of cocaine addiction.

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Introduction

Cocaine addiction constitutes a major health problem, and an estimated 1.6 million Americans meet the criteria for cocaine abuse or dependence (National Surveys on Drug Use and Health, 2006). Animal models for components of psychostimulant addiction have been well characterized (Ahmed and Koob, 1998; Kitamura et al., 2006) and mimic valid aspects of the loss of control over drug intake observed in humans (American Psychiatric Association, 2000; Koob, 2008). Specifically, cocaine self-administration with extended access [long access (LgA)] gradually increases over days, whereas the intake remains stable in rats with limited access [short access (ShA)] (Ahmed and Koob, 1998; Wee et al., 2007, 2008; Specio et al., 2008). This escalated drug intake is associated with increased breakpoints or responding for cocaine under a progressive ratio (PR) schedule, suggesting an enhanced motivation to seek cocaine and/or an enhanced efficacy of cocaine reward (Paterson and Markou, 2003; Wee et al., 2008) and

is also characterized by a withdrawal syndrome as measured by increased brain reward thresholds (Ahmed et al., 2002). Thus, investigations into the neuroadaptations that occur in LgA rats are invaluable for understanding the enhanced motivation to seek cocaine associated with the development of addiction.

The endogenous cannabinoid system is associated with aspects of drug addiction, as endocannabinoids (eCB) modulate the rewarding effects of ethanol, nicotine, opioids, and marijuana (for review, see Tanda and Goldberg, 2003; Lupica et al., 2004, Cohen et al., 2005; Gardner, 2005). Moreover, CB1 cannabinoid receptors are expressed in regions of the brain reward circuitry (Herkenham et al., 1990, 1991; Mailleux and Vanderhaeghen, 1992). However, the specific role of eCB in cocaine addiction remains widely inconclusive (Wiskerke et al., 2008). Whereas several studies suggest no role for CB1 receptors in cocaine self-administration per se (Fattore et al., 1999; Martin et al., 2000; Cossu et al., 2001; Lesscher et al., 2005; Caillé and Parsons, 2006; Filip et al., 2006; Caillé et al., 2007), other reports demonstrate a CB1 receptor involvement in both the acquisition and consolidation of cocaine reward (Soria et al., 2005; Xi et al., 2008) and in relapse to cocaine-seeking behavior (De Vries et al., 2001; Xi et al., 2006). However, the above studies evaluated the effects of CB1 receptor deletion/antagonism in animals given limited access to cocaine. It is unknown if long-term extended-access cocaine exposure could alter eCB–CB1 function.

We tested the hypothesis here that alterations in eCB transmission contribute to the increased motivation for cocaine associated with stimulant use. Thus, we examined the effects of systemic and intra-accumbal injections of the CB1 receptor antagonist SR141716A (Rimonabant) on cocaine self-administration under a PR schedule of reinforcement and showed a selective enhanced sensitivity in LgA rats compared with ShA rats. Additionally, LgA rats maintained an increased nucleus accumbens (NAc) eCB tone during cocaine self-administration compared with ShA rats and showed an upregulation of both phosphorylated (pCB1) and total CB1 receptor protein expression in the NAc and amygdala 24 h after last cocaine session. Results together suggest a dramatic increased activity of the eCB system in rats with extended access to cocaine self-administration.

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Materials and Methods

Animals.

Sixty-eight adult male Wistar rats (Charles River) weighting 225–250 g at the start of the experiment were used. Rats were housed two or three per cage with food and water available *ad libitum* and maintained in a reverse 12 h light/dark cycle, with lights on at 8:00 A.M. Twenty-three rats did not complete the experiments because of catheter failure or health complications. In three animals, the intravenous catheter was replaced for a new one which was implanted in the left jugular vein, and two animals were excluded of analysis because of misplaced cannula locations. All procedures were approved by The Scripps Research Institute Animal Care and Use Committee and were in accordance with National Institutes of Health (NIH) guidelines (NIH Publication no. 85-23, revised 1996).

Drugs and reagents.

Cocaine hydrochloride and SR141716A (Rimonabant) [*N*-piperidino-5-(4-chorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide] were generously provided by the National Institute on Drug Abuse. Cocaine HCl was dissolved in sterile 0.9% saline and filtered. SR141716A (Rimonabant) was dissolved in a vehicle of ethanol:emulphor:saline (1:1:18) by sonication for 30 s and was prepared fresh for every test session. Arachidonoylethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG), 1(3)-arachidonoylglycerol (1-AG), and (*S*)-(+)-arachidonyl-2′-hydroxy-1′-propylamide (*S*-2 methanandamide) were used as chromatographic standards for microdialysis and were purchased from Cayman Chemical. Rabbit anti-CB1 receptor (C1108) was purchased from Sigma. Rabbit polyclonal anti-pCB1 receptor (s.c.-17555) and mouse anti-β-tubulin (s.c.-53140) antibodies were purchased from Santa Cruz Biotechnology, goat anti-rabbit and goat anti-mouse IgG secondary antibodies were from Bio-Rad.

Intravenous surgery.

Rats were anesthetized with 2–3% of isoflurane mixed in oxygen and implanted with a sterilized SILASTIC catheter (0.64 inner diameter × 1.19 outer diameter mm; Dow Corning) into the right jugular vein under aseptic conditions. The distal end of the catheter was threaded under the skin to the back of the rat and exited the skin via a metal guide cannula (22G; Plastic One). Immediately after surgery, Flunixin (2.5 mg/kg, s.c.) was given as analgesic. The rats were subjected to antibiotic therapy (Timentin; 20 mg, i.v.) for at least 10 d. Catheters were flushed daily with heparinized saline (30 USP units/ml). The patency of catheters was tested using methohexital sodium (Brevital; 10 mg/ml, 2 mg/rat).

Cocaine self-administration procedure.

After at least 5 d of postoperative recovery, rats were trained to self-administer cocaine during daily 1 h sessions under a fixed ratio 1 (FR1) schedule of reinforcement in standard operant boxes (MedAssociates). The dose of cocaine (0.5 mg/kg/infusion) was prepared individually for each rat based on the body weight and updated every 2 or 3 d. Cocaine at 0.5 mg/kg/infusion accounts for a highly stable self-administration behavior in rats, and previous findings showed as well that the dose of 0.5 mg/kg/infusion of cocaine is within the range of the descending limb of the cocaine dose–response self-administration curve in rats (Xi et al., 2005; Wee et al., 2007, 2008), and thus this dose of cocaine is appropriate to measure reliable and stable dose-dependent effects. In addition, when LgA and ShA rats were compared using a dose/response test, there was an upward shift at all doses bracketing this dose (Ahmed and Koob, 1998, 1999, 2005; Deroche et al.,1999; Wee et al., 2007). The dose of cocaine was carefully chosen based on our and other's previous studies to reflect a dose where both increases and decreases in PR responding could be observed (Xi et al., 2005, 2008; Wee et al., 2007, 2008).

At the beginning of each session, rats were presented with two levers into the chamber. Responding on the right lever resulted in the 4 s delivery of 0.1 ml of cocaine and the illumination of a light above the lever that lasted throughout a time-out period of 20 s. Responding on the left lever was recorded but had no programmed consequences. Rats were trained to self-administer a higher cocaine dose (1 mg/kg/infusion) for 2 d before the dose of cocaine was reduced to 0.5 mg/kg/infusion over 14 subsequent baseline sessions. After this baseline period, the rats were divided into two groups, balanced by the average number of cocaine infusions per session during the last three baseline sessions. One group of rats were allowed to self-administer cocaine (0.5 mg/kg/infusion) during daily 6 h sessions (LgA), whereas the other group of rats was maintained on daily 1 h sessions (ShA) for the next 22 sessions. Cocaine self-administration sessions were conducted 6 d per week.

SR141716A (Rimonabant) pretreatment on cocaine self-administration.

After 22 escalation sessions, the effect of the CB1 receptor antagonist SR141716A was tested in LgA and ShA rats under a PR schedule of reinforcement. In the PR schedule, the response requirement began at one lever press/infusion and increased exponentially according to the following equation: lever press/infusion = $[5\times e^{(\text{infusion number}\times 0.2)}] - 5$ (Richardson and Roberts, 1996). The session length was limited to 12 h or ended when a rat failed to achieve the response requirement within 1 h. Test sessions (PR) were conducted 2 d per week and were preceded by two FR1 cocaine self-administration session days (LgA, 6 h session; ShA, 1 h session) to assure stable cocaine intake levels between the previous and the subsequent test session. Each rat received systemic vehicle pretreatments before four PR test sessions to habituate them to the injection procedure. Three doses of the CB1 antagonist SR141716A (0.3 mg/kg, 1.0 mg/kg and 3.0 mg/kg) and vehicle (see Drugs and reagents) were tested in a within-subject, Latin square design. The CB1 receptor antagonist was injected intraperitoneally 30 min before a test session. The SR141716A doses were chosen based on previous literature (Rodríguez de Fonseca et al., 1999; Navarro et al., 2001). Finally, each rat was again tested with vehicle injections in one last test session at the end of the study. No changes in responding to vehicle treatment were found on PR cocaine self-administration during and after the Latin square design.

Brain microdissection procedure.

Animals from the behavioral study were allowed to self-administer cocaine (LgA, 6 h session, $n = 6$; ShA, 1 h session, $n = 6$; FR1) during a

SR141716A wash-out period of at least 7 d after the last dose tested. For the purpose of comparison, age- and weight-matched drug-naive rats ($n = 4$) were included as a control group for the biochemical analyses. Twenty-four hours after the last escalation session rats were decapitated in random order under light isoflurane anesthesia. The brains were rapidly removed, snap frozen in 2-methylbutane, and stored at −80°C until dissection. On the day of dissection, brains were mounted and sliced in a cryostat (Cryocut 1800; Reichert-Jung, Leica). Brain tissue from the prefrontal cortex (PFC; +3.20 to +2.20 mm from bregma), NAc core (+2.20 to +0.70), NAc shell (+1.70 to + 0.70), dorsal striatum (DS; +1.70 to + 2.20), bed nucleus of the stria terminalis (BNST; −0.30 to −0.80), amygdaloid nucleus (Amy; −1.80 to −2.80), ventral hippocampus (VHp; −2.80 to −4.30), and dorsal hippocampus (DHp; −4.30 to −5.8) were punched out bilaterally in successive 500 μm sections with the assistance of a rat brain atlas (Paxinos and Watson, 1998). Punch needles were kept on dry ice during the procedure to prevent tissue from thawing within the needle, and brain punches were subsequently kept at −80°C until assay.

Western blot analysis of phosphorylated or total CB1 receptor and β-tubulin.

Brain punches were sonicated in a homogenization buffer containing 50 mm Tris-HCl, pH = 7.4, 10% w/v sucrose, 5 mm EDTA, 1 mm dithiotreitol, 1% w/v SDS, with Protease Inhibitor Mixture and Phosphatase Inhibitor Cocktails I and II diluted 1:100 each (Sigma). Protein content was determined in homogenized samples using a detergent-compatible protein assay (Bio-Rad). Equal amounts of protein (15 μg) were loaded per lane including 3–5 animals per group in each gel. In a separate experiment, equal amounts of protein (15 μg) from various brain structures were loaded to compare basal brain-wide CB1 receptor and pCB1 immunoreactivies in naive animals. Proteins were separated by 10% SDS-PAGE using Tris-Glycine-SDS buffer system (Bio-Rad) and transferred to polyvinylidene membranes (GE Healthcare). Membranes were then blocked for 2 h in PBS containing 5% nonfat milk to reduce nonspecific binding of antibodies, followed by an overnight incubation at 4°C with polyclonal rabbit anti-CB1 (1:500 dilution) or anti-pCB1 (Ser 317) (1:250 dilution) and mouse anti-β-tubulin (1:2500 dilution). Membranes were washed and then incubated for 1 h with species-specific peroxidase-conjugated secondary antibody (1:10000 dilution; Bio-Rad). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) Western blot detection system (GE Healthcare), following by exposure to Amersham Hyperfilm ECL for 1–5 min. Different film exposure times were used to ensure that bands were not saturated, and immunoreactivity was quantified by ImageJ software (NIH). The anti-pCB1 antibody used in this study was raised against the CB1 receptor, which is phosphorylated by PKC at Ser 316 (humans)/Ser 317 (rodents) (Matsuda et al., 1990; Gérard et al., 1991; Garcia et al., 1998). The antibody recognized two bands corresponding to the glycosylated (63 kDa) and the nonglycosylated (53 kDa) protein, both reflecting functional pCB1 receptor protein (Howlett et al., 1998; Porcella et al., 2000). Both glycosylated and nonglycosylated phosphorylated forms were quantified and expressed as total pCB1 protein. The antibody for total CB1 receptor density recognized one band of 64 kDa corresponding to the monomeric form of the fully glycosylated receptor (Fusco et al., 2004) that was quantified and expressed as total CB1 receptor protein [the antibody recognized also one nonspecific band of 33 kDa, which is due probably to the secondary antibody used; for details, see Fusco et al. (2004)]. β-Tubulin was used as a loading control and so the immunoreactivity of CB1 and pCB1 receptors was corrected for β-tubulin levels.

Intracerebral microdialysis cannula implantations.

To investigate cocaine's regulation of eCB levels in LgA and ShA rats during cocaine self-administration, another group of rats was trained as described above. After the escalation period (22 sessions), rats were anesthetized (2–3% isoflurane) and implanted with a guide cannula (SciPro) aimed at the NAc shell using a stereotaxic frame with the tooth bar at −3.3 mm below the interaural zero. Coordinates for the shell cannula implantation were as follows: +1.6 mm from bregma, ±0.8 mediolateral, and −5.7 mm below dura (Paxinos and Watson, 1998). Cannulae were implanted in the right or left NAc shell of the rats in random order. To avoid disruption in animal behavior resulting from surgery, rats were allowed to self-administer cocaine during at least six more sessions before the microdialysis experiment.

Probe implantation and *in vivo* microdialysis.

For probe implantation, each animal was lightly anesthetized (1–2% isoflurane), and the dialysis probes (2 mm polyethyl sulfone membrane, 15 kDa MW cutoff; SciPro) were inserted in the guide cannulae and secured with a light layer of cement (Double/Bubble Epoxy; Hardman). The probes were perfused overnight with artificial CSF (0.1 μl/min) composed of the following: 149 NaCl nm, 2.8 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 0.25 ascorbic acid, and 5.4 d-glucose. The morning of the microdialysis experiment, the artificial CSF was replaced by an artificial CSF solution containing 30% (w/v) hydroxypropyl-β-cyclodextrin, and probes were perfused for 90 min at 0.6 μl/min flow. Subsequently, dialysis samples were collected at 15 min intervals during the baseline period (90 min) and subsequent operant cocaine self-administration (90 min). Animals remained in the same cage and room during the microdialysis perfusion and were transferred to the operant chambers in the same room exclusively for cocaine self-administration.

Liquid chromatography/mass spectrometry analysis of dialysate eCB content.

Dialysate levels of AEA, 2-AG, and 1-AG were determined using high-performance liquid chromatography coupled with electrospray ionization mass spectrometry. 2-AG levels were calculated by summing 1-AG and 2-AG peak areas to control for 2-AG degradation over time. The procedure used has been previously described in detail (Caillé et al., 2007).

Intracerebral infusion cannulas.

Animals were trained as described in the cocaine self-administration procedure. After 24 escalation sessions, rats were anesthetized (2–3% isoflurane) and implanted with bilateral microinfusion guide cannula (22 gauge, 12 mm length, stainless steel) that terminated 2 mm above the surface of the NAc (from bregma: anterior–posterior, +1.7, medio–lateral, ±0.9, dorso–ventral, −5.4 from dura) (Paxinos and Watson, 1998). After postoperative recovery, rats were allowed to self-administer cocaine until similar self-administration rates to those before surgery were established before the SR141716A testing.

Intra-NAc SR141716A (Rimonabant) testing on PR cocaine self-administration.

The effects of local SR141716A administration on cocaine self-administration under a PR schedule were evaluated in a separate group of animals. Animals were trained for responding in a PR schedule during a total of four sessions (two sessions before intracerebral surgery and two sessions after surgery) which were always preceded by cocaine FR1 sessions. After establishment of stable self-administration behavior, animals received an initial sham microinjection (insertion but no liquid infusion) immediately before a PR cocaine session to acclimate them to the procedure and to produce the initial tissue damage from injection insertion. Subsequently, vehicle and SR141716A (1.0 and 3.0 μg per side) were injected via bilateral 33 gauge microinjections that extended 2 mm beyond the tip of the guide cannulas. Infusions of 0.5 μl per side were made over a 2 min period, followed by an additional 1 min to allow drug diffusion before injector removal. The doses of SR141716A for intracerebral microinjections were chosen on the basis of a ratio of ~1000:1 for systemic/intracerebral microinjection dosing (Xi et al., 2006) and after our previous work with SR141716A (Caillé et al., 2007). Intra-NAc administration of SR141716A was tested immediately before cocaine PR sessions in a counter balance order across the rats, and each dose was tested only once in each animal.

Histology.

Microdialysis probe locations were examined after completion of the experiments in all the animals. Rats were killed by decapitation and brains removed and rapidly frozen on dry ice. Brains were coronally sliced in a cryostat, and the probe placements were verified with the help of a rat atlas (Paxinos and Watson, 1998).

Cannula placements for intra-NAc infusions were examined as follows: frozen coronal brain section (60 μm) were dehydrated and rehydrated in ethanol, stained in 0.1% cresyl violate acetate for 10 min, and coverslipped for histological verification of the implant location under a light microscope.

Statistical analyses.

Data were analyzed using GraphPad Prism version 4.0 (GraphPad Software). Cocaine intake was expressed as the mean number of infusions in both

the first hour of the session and in the total session for each group of rats. Cocaine self-administration was compared across daily sessions over the initial 22 sessions using repeated-measures two-way ANOVA followed by a Bonferroni *post hoc* test (access times daily session). The effect of pretreatment of SR141716A on cocaine PR self-administration was compared between LgA and ShA rats using repeated-measures two-way ANOVA with cocaine access as the between-subjects factor and SR141716A dose as the within-subjects factor followed by the Bonferroni post-test. Additionally, when a significant access times dose interaction was found, one-way ANOVA followed by Newman–Keuls *post hoc* test was conducted on simple main effects, and Student's *t* test was performed on the appropriate pairing (vehicle-injected rats). To identify possible rate-dependent effects, a median split analysis was performed on the LgA animal's response under a PR schedule (cocaine injections per session) that yielded high and low subgroups, which were compared using an independent samples *t* test. Subsequently, the percentage change from baseline after SR141716A administration was compared for the high and low responding groups using an independent samples *t* test. Differences in baseline dialysate AEA and 2-AG concentrations between LgA and ShA rats were analyzed by two-tailed Student's *t* test. Subsequently, dialysate AEA and 2-AG levels were expressed as a percentage of the average six dialysate baseline concentrations for each group. Changes in dialysate sample content between groups were investigated measuring the area under the curve (AUC) for AEA and 2-AG of the transformed data. The AUC was calculated for each animal by subtracting each percentage of baseline data points from 100 and summing all data points collected during the experimental period. Calculated cumulative AUC was then compared between groups by Student's *t* test for AEA and 2-AG. Additionally, repeated-measures two-way ANOVA analysis of the calculated data was used to investigate interaction between groups. Percentage of changes from baseline levels in each group were reported by using repeated-measures one-way ANOVA after Newman–Keuls multiple comparison test when a significant interaction or main effect was found.

Data from Western blot analyses were expressed as a percentage of the drug-naive control group and were analyzed using one-way ANOVA followed by Newman–Keuls multiple comparison test when a significant F value was obtained.

Go to:

Results

Cocaine self-administration under a fixed-ratio schedule in ShA and LgA rats

CB1 receptor antagonist group

A two-way ANOVA found a significant interaction between group and daily session in escalation of cocaine self-administration (0.5 mg/kg/infusion) within a whole session ($F_{(21,374)} = 7.27$, $p < 0.0001$) as well as during the first hour of each session ($F_{(21,374)} = 3.08$, $p < 0.0001$). Additionally, there were main effects of group ($F_{(1,374)} = 2561$, $p < 0.0001$; first hour $F_{(21,374)} = 169.7$, $p < 0.0001$) and daily session ($F_{(21,374)} = 7.58$, $p < 0.0001$; first hour $F_{(21,374)} = 3.85$, $p < 0.0001$) on cocaine self-administration levels. Subsequent analyses revealed that cocaine self-administration in ShA rats ($n = 10$) remained stable over 22 sessions (Fig. 1*a*,*b*, open circles). However, LgA rats ($n = 9$) self-administered more cocaine over time, with the increase becoming evident in session five (within the whole session), and in session six (during the first hour), compared with respective cocaine intake in session one (Fig. 1*a*,*b*, filled circles). Escalated cocaine self-administration by LgA rats was maintained over the course of the study.

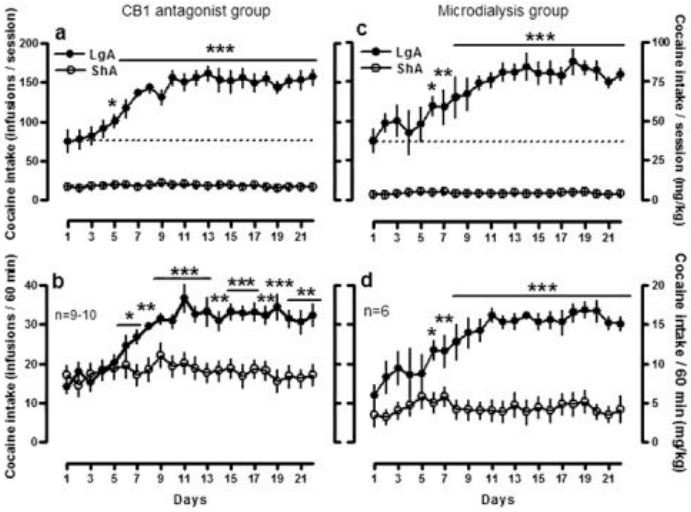

Figure 1.

Cocaine self-administration under an FR1 schedule during the escalation period. Left panels (***a***, ***b***) are data from animals in the CB1 receptor antagonist study, and right panels (***c***, ***d***) are data from animals in the microdialysis study. Top panels (***a***, ***c***) are data from the entire 6 h sessions, and bottom panels (***b***, ***d***) are data from the first hour of the sessions. Filled circles correspond to cocaine (0.5 mg/kg/infusion) extended-access rats (6 h session, LgA), and open circles correspond to cocaine limited-access rats (1 h session, ShA). Data are expressed as the mean ± SEM of the number of cocaine infusions on the left axis and mg/kg on the right axis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with session one.

Endocannabinoid microdialysis group

Similarly, in the microdialysis study, LgA rats ($n = 6$) showed an escalation in the cocaine intake over 22 sessions, compared with session one, within the 6 h session (Fig. 1*c*, filled circles) and during the first hour of each session (Fig. 1*d*), which started in session number six. A two-way ANOVA found a significant interaction between group and daily session in cocaine self-administration within session ($F_{(21,220)} = 3.61$, $p < 0.0001$) and during the first hour session ($F_{(21,220)} = 2.446$, $p < 0.001$). Additionally, there were main effects of group ($F_{(1,220)} = 1406$, $p < 0.0001$; first hour $F_{(1,220)} = 400.6$, $p < 0.0001$), and daily session ($F_{(21,220)} = 3.61$, $p < 0.0001$; first hour $F_{(1,220)} = 2.44$, $p < 0.0001$) in the levels of cocaine self-administration. Self-administration in the ShA group ($n = 6$) remained stable over the 22 sessions (Fig. 1*b*,*d*, open circles).

Effect of CB1 receptor antagonist SR141716A (Rimonabant) on cocaine self-administration under a PR schedule in LgA and ShA rats

To determine whether CB1 activation drives the increased motivation for cocaine observed in dependent animals, we tested a CB1 antagonist on cocaine self-administration under a PR schedule of reinforcement. LgA rats responded ~228 times at the active lever (cumulative response) to achieve breakpoint of 52.79 ratio/infusion (12.67 reinforcements) (Fig. 2, left panel). ShA rats responded ~97 times to achieve an average breakpoint of 25.83 (9.3 reinforcements) (Fig. 2, right panel). The breakpoint for cocaine (0.5 mg/kg/infusion) in vehicle-injected rats was significantly higher in LgA rats versus ShA rats (Student's *t* test, $^{\#}p < 0.01$).

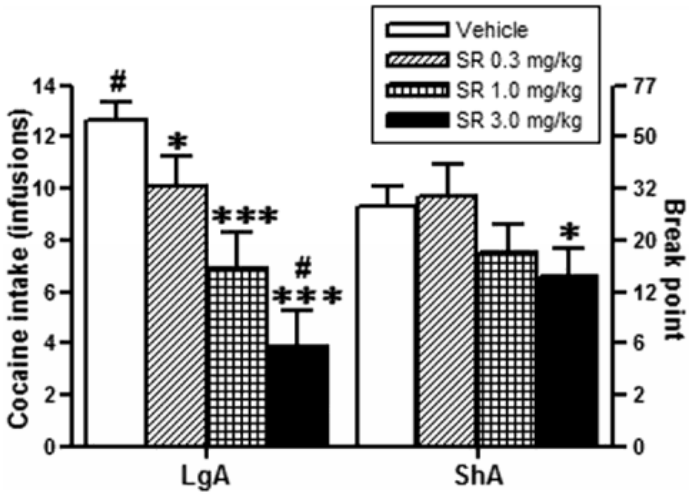

Figure 2.

Effects of systemic CB1 receptor antagonist SR141716A on the breakpoint for cocaine in LgA and ShA rats. SR141716A (SR, Rimonabant) was injected (intraperitoneally) 30 min before the test session. Sessions under a PR schedule ended when rats did not achieve cocaine (0.5 mg/kg/infusion) reinforcement within 1 h. Data are expressed as the mean ± SEM of the number of infusions per session on the left axis and the breakpoint (maximal ratio per infusion) on the right axis. Bars on the left side are data from cocaine extended-access (LgA) rats, and bars on the right side are data from limited-access (ShA) rats. Responding for cocaine was different under a PR schedule in vehicle-injected rats between the access conditions: $^{\#}p < 0.01$ compared with ShA. SR141716A (0.3 and 1.0 mg/kg) decreased the breakpoint for cocaine selectively in LgA rats compared with vehicle-injected animals; the highest dose of SR141716A (3.0 mg/kg) had a significant effect in both LgA and ShA rats compared with their respective controls: $^{*}p < 0.05$, $^{***}p < 0.001$ compared with vehicle-injected rats in the same access condition. Different from ShA vehicle-injected rats: $^{\#}p < 0.01$.

Figure 2 shows the effects of pretreatment with SR141716A (0.3, 1.0 and 3.0 mg/kg, i.p.) on the breakpoint for cocaine in both long and short access conditions. A two-way ANOVA between SR141716A pretreatment and cocaine access showed a significant interaction between both factors ($F_{(3,51)} = 5.80$, $p = 0.0017$) and a main effect of SR141716A on cocaine breakpoint ($F_{(3,51)} = 23.49$, $p < 0.0001$). Additional post-test revealed a significant reduction in the breakpoint for cocaine in LgA rats at all doses of SR141716A tested versus vehicle in the same access condition (Fig. 2) (0.3 mg/kg, $^{*}p < 0.05$; 1.0 and 3.0 mg/kg, $^{***}p < 0.001$). Similar analysis in ShA rats showed that pretreatment with 0.3 and 1.0 mg/kg doses of SR141716A did not modify the breakpoint for cocaine compared with vehicle-injected rats in the same access condition [$p > 0.05$, nonsignificant (n.s.)], and only the highest dose (3.0 mg/kg) had a significant effect in the limited access animals ($^{*}p < 0.05$). Furthermore, even with the lowest dose of SR141716A, there was no longer a significant difference between LgA and ShA rats in responding for cocaine ($p > 0.05$, n.s.). One-way ANOVA analyses identified a main effect of SR141716A dose in LgA and ShA rats ($F_{(3,24)} = 18.42$, $p < 0.0001$ and $F_{(3,27)} = 5.24$, $p < 0.01$, respectively) and *post hoc* tests confirmed a reduction in cocaine breakpoint in LgA rats at 1.0 and 3.0 mg/kg SR141716A dose ($p < 0.001$) and in ShA rats at 3.0 mg/kg dose ($p < 0.05$), compared with the respective vehicle-injected animals. Finally, SR141716A at 3.0 mg/kg decreased cocaine breakpoints in the LgA group to even lower ratios than ShA vehicle-treated rats (*t* test, $p < 0.01$). This fact suggests that high doses of SR141716A may interact with neuroadaptative changes in the LgA group that are not present in the ShA group and/or that CB1 receptor signaling provides a general influence on the motivational effects in LgA animals beyond simply conferring the increased cocaine intake and/or breakpoints observed in LgA versus ShA animals.

To address the possibility that higher response rates would be more susceptible to pharmacological modulation by the CB1 antagonist, we conducted a median split analysis on the LgA animals' response rates. A median split analysis of the response of the LgA animals in a PR schedule (cocaine injections per session) yielded high and low responder subgroups with mean lever-presses (±SEM) 14.6 ± 0.25 and 11.83 ± 0.79, respectively. SR141716A induced equivalent decreases in percentage change from baseline at all doses tested regardless of whether the animals had high or low response rates initially. For example, SR141716A at dose of 1 mg/kg produced 41.1 ± 9.98% and 43.63 ± 11.41% decrease in responding in the high and low responders, respectively. This further supports the hypothesis that SR141716A acted independently of response rate and was more potent in LgA animals.

The amount of cocaine in the rat's system during testing was calculated based on data in Figure 2 and is represented in Table 1. Analysis of the cocaine intake data presented in Table 1 shows that on the test days there were no robust differences in the amount of cocaine ingested between the two groups. For example, there are no differences in the amount of cocaine in the rat's system on the test days after SR141716A 0.3 and 1.0 mg/kg pretreatment (5.06 mg/kg vs 4.85 mg/kg and 3.45 mg/kg vs 3.75 mg/kg, respectively). However, during those test days, SR141716A decreased responding selectively in the LgA group and not in the ShA group (Fig. 2). This supports the hypothesis that SR141716A acted independently of the amount of cocaine currently in the rat's system during testing. Rather, the data suggest that SR141716A altered the motivation for obtaining cocaine in the LgA group.

Table 1.

Cocaine intake (milligrams per kilograms) by LgA and ShA rats during systemic SR141716A testing (PR sessions)

	Cocaine (mg/kg), LgA	Cocaine (mg/kg), ShA	*p* value
SR vehicle	6.33 ± 0.34	4.65 ± 0.40*	< 0.01
SR 0.3 mg/kg	5.06 ± 0.58	4.85 ± 0.62	n.s.
SR 1.0 mg/kg	3.45 ± 0.70	3.75 ± 0.55	n.s.
SR 3.0 mg/kg	1.95 ± 0.69	3.30 ± 0.53	n.s.

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The amount of cocaine ingested by LgA and ShA rats on the test days was calculated based on data in Figure 2. Data in the table are represented as mean ± SEM. Analysis of the cocaine intake paired data presented in the table (LgA vs ShA; *t* test) shows differences in the amount of cocaine between vehicle-injected LgA and ShA animals and no differences in the amount of cocaine ingested between the two groups on the other test days. These data support the hypothesis that SR141716A acted independently of the amount of cocaine currently in the rat's system during testing. SR, SR141716A; different from cocaine LgA in the same condition: $^{*}p < 0.01$; n.s., nonsignificant.

Endocannabinoid levels during cocaine self-administration in the NAc shell of rats with a history of LgA and ShA to

cocaine

To test whether eCB transmission is differentially affected by extended versus limited access to cocaine, we measured eCB levels during cocaine self-administration in LgA and ShA rats using *in vivo* microdialysis. AEA and 2-AG levels (see Materials and Methods) were measured in the NAc shell, where changes in eCB transmission were reported previously after heroin and ethanol self-administration (Caillé et al., 2007).

Baseline AEA and 2-AG levels are depicted in Figure 3*d*. No differences in the baseline levels of either AEA or 2-AG were found between ShA and LgA rats (Student's *t* test, $p > 0.05$, n.s.), although AEA baseline levels in the LgA group were relatively lower (~37%) compared with the ShA group (2.89 ± 0.33 vs 1.80 ± 0.51 nm, $p > 0.05$, n.s.). As expected, LgA rats self-administered more cocaine than ShA rats during the 90 min microdialysis session (Student's *t* test, $p < 0.01$). Total cocaine intake during the test session was 21.63 ± 5.67 mg/kg for LgA rats and 5.33 ± 1.28 mg/kg for ShA rats. Figure 3*c* shows the cocaine response record for a representative animal in each group. AUC analysis revealed a significant difference in AEA levels between LgA and ShA rats during the entire session (Fig. 3*a*) [Student's *t* test (AUC), $^{*}p < 0.05$]. Two-way ANOVA revealed a significant interaction between group and cumulative AUC over time for AEA levels ($F_{(6,48)} = 4.30$, $p \leq 0.01$) and main effects of group ($F_{(1,48)} = 6.30$, $p < 0.05$) and time ($F_{(6,48)} = 17.79$, $p < 0.0001$) in the AEA dialysate. Further analyses revealed that cocaine self-administration in ShA rats produced rapid decreases in AEA (Fig. 3*a*) (repeated-measures one-way ANOVA, $^{\#}p < 0.05$), whereas AEA levels in the LgA group remained similar to baseline during the 90 min session (see Fig. 6*a*) (repeated-measures one-way ANOVA, $p > 0.05$, n.s.). Regarding 2-AG levels, no statistical difference in the AUC analysis was found between groups (Fig. 3*b*) [Student's *t* test (AUC), $p > 0.05$, n.s.]. Two-way ANOVA analyses of 2-AG dialysate failed to find an interaction between group and the cumulative AUC over time ($F_{(6,48)} = 1.02$, $p > 0.05$, n.s.), although there was a main effect of time in the 2-AG levels ($F_{(6,48)} = 10.27$, $p < 0.0001$). Further analyses revealed decreases in 2-AG levels over time in the ShA group (Fig. 3*b*) (repeated-measures one-way ANOVA, $^{\#}p < 0.05$) but no significant effect in the LgA group (Fig. 3*b*) (repeated-measures one-way ANOVA, $p > 0.05$, n.s.).

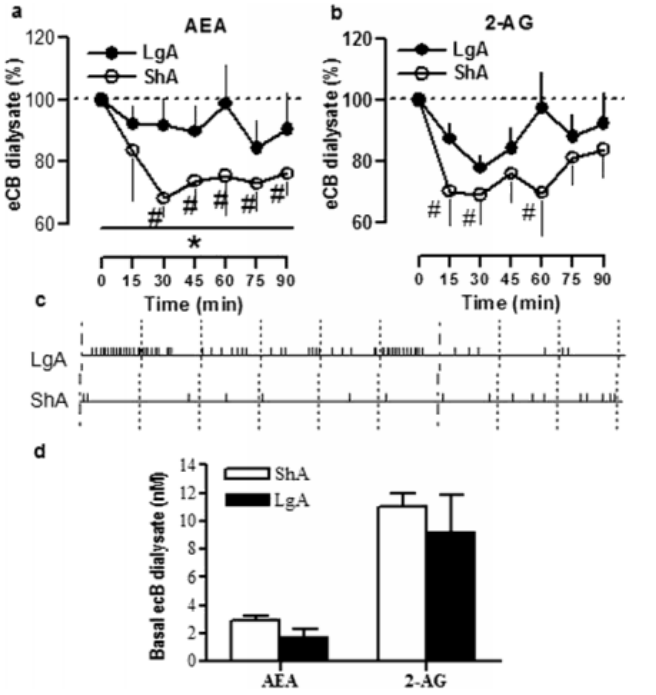

Figure 3.

In vivo microdialysis for AEA and 2-AG in the NAc shell of LgA and ShA rats during cocaine self-administration. NAc shell eCB dialysates were collected every 15 min in cocaine LgA (filled circles) and ShA (open circles) rats. Time 0 represents basal levels for AEA (***a***) and 2-AG (***b***), which were calculated as mean of the six dialysated samples collected before the self-administration session. Values are expressed as percentage of the baseline samples ±SEM. Differences in the AUC between LgA and ShA: $^{*}p < 0.05$. Different from baseline levels in the same condition: $^{\#}p < 0.05$ (one-way ANOVA, Newman–Keuls test). Central panel (***c***) represents the response records for representative dialysated LgA and ShA animals during the cocaine self-administration test session. Small marks represent an intravenous infusion of cocaine, and larger marks represent 10 min periods within the session. Basal concentrations for AEA and 2-AG are depicted in ***d***.

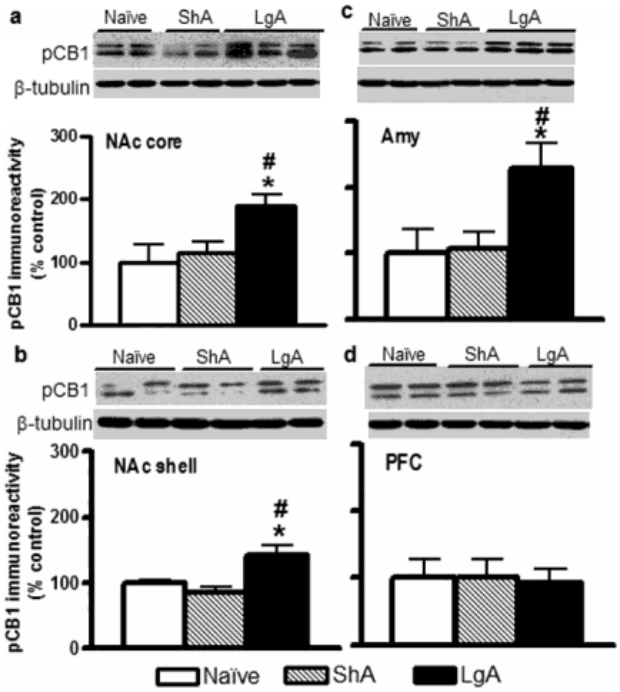

Figure 6.

Western blot analysis of pCB1 receptor immunoreactivities in NAc core, NAc shell, Amy, and PFC of drug-naive and cocaine ShA and LgA rats. Top panels show representative immunoblots for pCB1 and the loading control β-tubulin in nucleus accumbens core (***a***; NAc core), nucleus accumbens shell (***b***; NAc shell), Amy (***c***), and PFC (***d***). Bar graphs show the quantification of pCB1 receptors immunoreactivity (double-band quantification; see Materials and Methods). Data in graphs are represented as mean ± SEM ($n = 4–5$) and expressed as percentage values of the drug-naive rats for each group in every brain structure after normalization by β-tubulin. Animals were killed 24 h after the last cocaine session. LgA, Long access to cocaine (filled bars); ShA, short access to cocaine (striped bars); Naïve, drug-naive rats (open bars). Different from drug-naive rats, $^{*}p < 0.05$; different from ShA rats, $^{\#}p < 0.05$. β-Tubulin expression was constant across the groups ($p > 0.05$, n.s.).

Histology

The active portion of the dialysis membrane was located in the NAc shell between 1.6 and 1.7 mm anterior to bregma in all cases ($n = 10$) (Fig. 4).

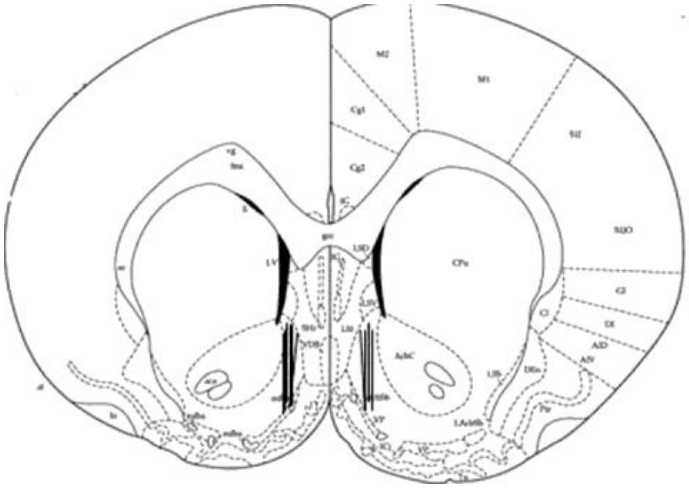

Figure 4.

Schematic representation of the microdialysis probe placements. The depicted section represents a coronal brain slice 1.6 mm anterior to bregma (Paxinos and Watson, 1998). Vertical bars correspond to the location of the active microdialysis membrane (2 mm) within the NAc shell. Cannulae for probe insertion were implanted in the right or left NAc shell of the rats in random order ($n = 10$).

Cannula placements for intra-accumbal infusions were located in the NAc between 2.28 and 2.76 mm anterior to bregma (see Fig. 9). Two animals (one LgA and one ShA rats) had misplaced cannulas, and therefore, only rats with cannulas implanted in the NAc shell were included in the analysis ($n = 10$).

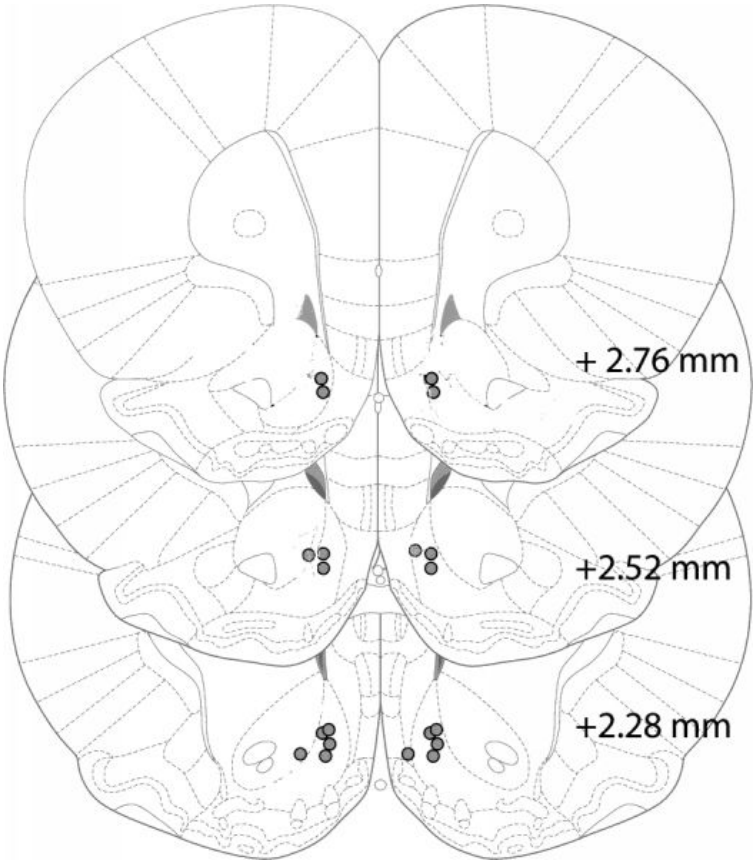

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Figure 9.

Schematic representation of cannula implants for intra-NAc infusions. Placements of each drug microinjector in the NAc are depicted in the coronal sections. Each brain section represents a coronal slice 2.76, 2.52, or 2.28 mm anterior to bregma (Paxinos and Watson, 1998), and each circle corresponds to the location of the microinjector tip within the NAc (n = 10). Animals with cannula implants outside of the NAc were excluded from statistical analysis.

CB1 and pCB1 receptor distribution in the brain

Figure 5 shows brain-wide total CB1 and phosphorylated CB1 receptor distribution in a representative drug-naive animal. We observed that functional CB1 receptors are widely distributed in the brain and concentrated in addiction-related brain areas such as the NAc core and shell, amygdala, bed nucleus of the stria terminalis, and prefrontal cortex, in agreement with other reports (Herkenham et al., 1990, 1991; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998; Hermann and Lutz, 2005). The pattern of CB1 receptor expression slightly differs from the pCB1 levels, with high density of both proteins observed in prefrontal cortex, dorsal striatum, and hippocampus, in accordance with the well known regulatory effect of CB1 receptors on many other processes in the brain, including locomotion and memory. The bar graph in Figure 5 represents the quantification of total CB1 receptor and pCB1 receptor density (see Materials and Methods) normalized by β-tubulin levels (51 kDa) in each brain area and expressed as a ratio (Fig. 5).

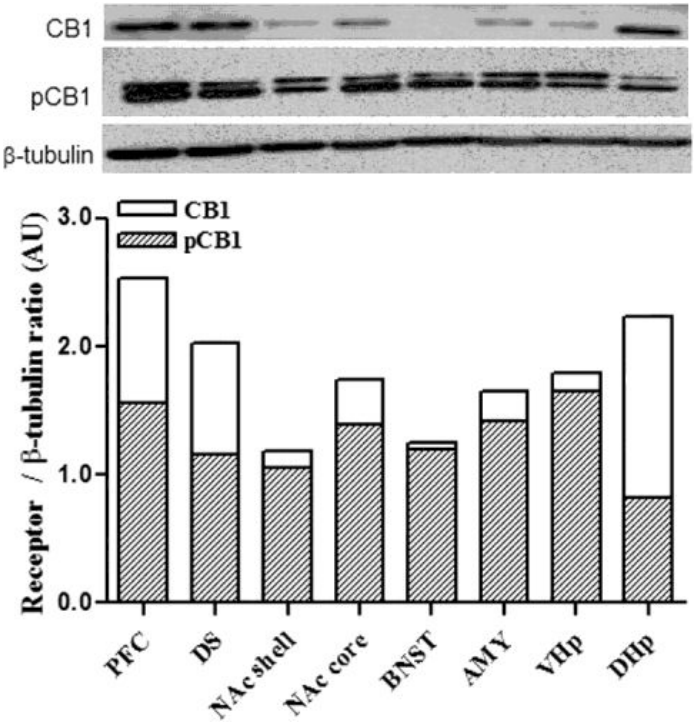

Figure 5.

Regional differences in total CB1 receptor and pCB1 protein expression in the brain. Top, Representative immunoblots for total CB1, pCB1 receptors, and the loading control β-tubulin show the pattern of functional CB1 receptor distribution throughout the brain. Bottom, The bar graph shows the quantification of the single band corresponding to CB1 receptor protein expression and the double band (see Materials and Methods) corresponding to pCB1 receptor immunoreactivity, both normalized by β-tubulin immunoreactivity. Data are expressed as receptor/β-tubulin immunoreactivity ratio. AU, Arbitrary units.

Increased phosphorylated and total CB1 receptor protein expression in the amygdala and NAc of cocaine LgA rats

High densities of phosphorylated CB1 receptor protein were observed in LgA rats compared with ShA and drug-naive rats specifically in the NAc core ($F_{(2,10)} = 6.25$, $p < 0.05$), NAc shell ($F_{(2,11)} = 6.45$, $p < 0.05$) and the amygdala ($F_{(2,11)} = 4.50$, $p < 0.05$) 24 h after the last cocaine escalation session (Fig. 6) (Newman-–Keuls multiple comparison test: $^{\#}p < 0.05$ compared with ShA; $^{*}p < 0.05$ compared with drug-naive rats). In contrast, no changes in pCB1 immunoreactivity were found in other brain structures such as the prefrontal cortex (Fig. 6), bed nucleus of the stria terminalis, dorsal striatum, and hippocampus (data not shown). There were no differences in pCB1 immunoreactivity between ShA and drug-naive rats ($p > 0.05$, n.s.) in any of the brain structures tested (Fig. 6). Moreover, cocaine self-administration did not change β-tubulin levels in any region tested (Fig. 6).

To elucidate whether pCB1 receptor increases reflected changes in total CB1 receptor densities, we analyzed total CB1 receptor immunoreactivity across the groups in the brain structures where we found an increase in pCB1 receptor (Fig. 7). Total CB1 receptor protein was increased in LgA rats in the NAc core ($F_{(2,12)} = 4.851$, $p < 0.05$), the NAc shell ($F_{(2,12)} = 4.225$, $p < 0.05$) and the amygdala ($F_{(2,16)} = 5.483$, $p < 0.05$) 24 h after the last cocaine escalation session (Fig. 7) (Newman-–Keuls multiple comparison test: $^{\#}p < 0.05$ compared with ShA; $^{*}p < 0.05$ compared with drug-naive rats). No changes in CB1 receptor were observed between ShA and drug-naive rats ($p > 0.05$, n.s.). Whereas most drugs of abuse alter CB1 receptor density in different directions (Basavarajappa et al., 1998; Gonzalez et al., 2002), no changes were observed after noncontingent cocaine administration in limbic forebrain (Gonzalez et al., 2002), which is in agreement with the lack of receptor changes observed in our ShA rats. However, we observed increased CB1 receptor density specifically in cocaine dependent animals, and similarly, opioid- and ethanol-dependent animals showed a permanent CB1 receptor upregulation in reward-related areas and enhanced sensitivity to reward disruption induced by cannabinoid receptor antagonists (Rodríguez de Fonseca et al., 1999; Navarro et al., 2001, 2004; Rimondini et al., 2002).

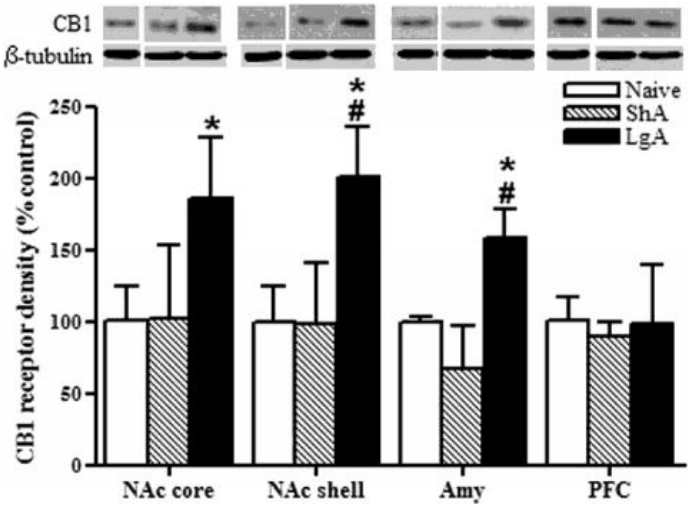

Figure 7.

Upregulation of total CB1 receptor density in NAc and Amy of LgA rats. Quantification of total CB1 receptor density in the NAc core, NAc shell, Amy, and PFC of LgA (long access; filled bars), ShA (striped bars), and Naive (drug-naive; open bars) animals. Data in graphs are corrected for β-tubulin, expressed as percentage values of the drug-naive rats, and represented as mean ± SEM ($n = 3$–6). Animals were killed 24 h after the last cocaine session. Different from ShA animals, $^{*}p < 0.05$; different from ShA rats, $^{\#}p < 0.05$.

Intra-NAc infusion of the CB1 receptor antagonist SR141716A reduces cocaine PR responding selectively in LgA rats

To confirm the hypothesized mechanism for the systemic effects of SR141716A, we performed an additional experiment where we infused bilaterally the CB1 antagonist SR141716A into the NAc immediately before a cocaine PR session. Rats were previously trained under an FR1 schedule in conditions of limited or extended access (see Materials and Methods), and cocaine self-administration data (Fig. 8*b*,*c*) were analyzed by a mixed-factor two-way ANOVA. The analysis identified a significant interaction between group and daily session within a whole session (Fig. 8*b*) ($F_{(23,230)} = 3.08$, $p < 0.0001$) and during the first hour of each session (Fig. 9*c*) ($F_{(23,230)} = 1.78$, $p = 0.02$). Additionally, there were main effects of group ($F_{(1,230)} = 14.12$, $p = 0.004$), and daily session ($F_{(23,230)} = 3.79$, $p < 0.0001$; first hour $F_{(23,230)} = 4.74$, $p < 0.0001$) on cocaine self-administration levels. Subsequent analyses revealed that cocaine self-administration in ShA rats ($n = 6$) remained stable over 24 sessions (Fig. 8*b*,*c*, open circles). However, LgA rats ($n = 6$) self-administered more cocaine over time, with the increase becoming evident in session nine, compared with respective cocaine intake in session one (Fig. 9*a*,*b*, filled circles).

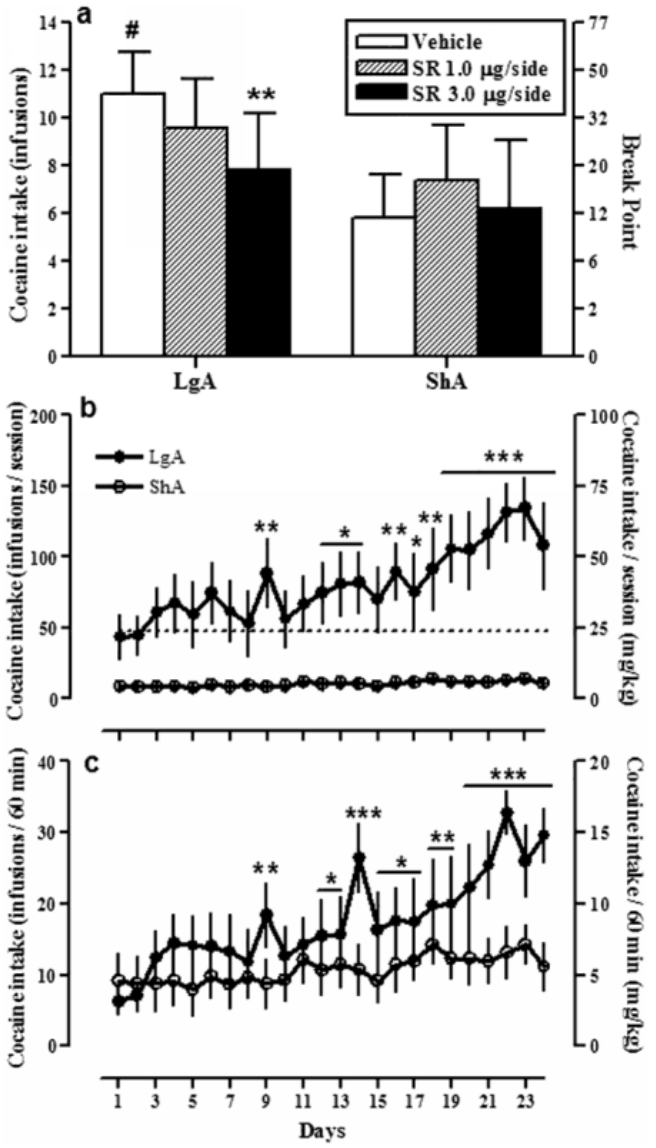

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Figure 8.

Intra-NAc infusions of SR141716A reduce cocaine breakpoints in LgA but not ShA rats. SR141716A (SR, Rimonabant) was infused locally into the NAc shell immediately before the test session (***a***). Sessions under a PR schedule ended when rats did not achieve cocaine (0.5 mg/kg/infusion) reinforcement within 1 h. Data in ***a*** are expressed as the mean ± SEM of the number of cocaine infusions per session on the left axis and the breakpoint (maximal ratio per infusion) on the right axis. Bars on the left side (***a***) are data from cocaine extended-access (LgA) rats, and bars on the right side are data from limited-access (ShA) rats. Bilateral infusions of SR141716A (3.0 µg/side) decreased the breakpoint for cocaine selectively in LgA rats compared with vehicle-injected animals. Different from vehicle-injected rats in the same access condition, $^{**}p < 0.01$. Different from ShA vehicle-injected rats, $^{\#}p < 0.05$. Cocaine self-administration data (FR1) during the entire session (***b***) and the first hour of the session during the escalation period (***c***) are represented in the bottom graphs. Filled circles represent cocaine (0.5 mg/kg/infusion) extended-access rats (6 h session, LgA), and open circles correspond to cocaine-limited-access rats (1 h session; ShA). Data are expressed as the mean ± SEM of the number of cocaine infusions on the left axis and milligrams per kilograms on the right axis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with session one.

Under a PR schedule, LgA rats achieve an average breakpoint of ~48 ratio/infusion (11.0 reinforcements) (Fig. 8*a*, left side), whereas ShA rats responded achieve an average breakpoint of ~12 ratio/infusion (5.8 reinforcements) (Fig. 8*a*, right side). The breakpoint for cocaine in vehicle-injected rats was significantly higher in LgA rats versus ShA rats (Student's *t* test, $^{\#}p < 0.05$). Figure 8*a* shows the effects of bilateral infusions of SR141716A (0.0, 1.0, and 3.0 µg/side; 0.5 µl per side in 2 min) on the breakpoint for cocaine in both long and short access conditions. A two-way ANOVA between SR141716A pretreatment and cocaine access showed a significant interaction between both factors ($F_{(2,16)} = 4.56$, $p = 0.027$). Additional analysis revealed that bilateral SR141716A accumbal infusions reduced breakpoints for cocaine selectively in LgA rats (Fig. 8*a*, left panel) ($F_{(2,8)} = 12.87$, $^{**}p < 0.01$), and no effect was observed in the ShA group (Fig. 8*a*, right panel) ($p > 0.05$, n.s.).

A potential confound is that the site-specific injections may diffuse to other surrounding brain regions. Thus, the degree of spread of the antagonist was determined by an injection of 0.5 µl of cresyl violet using the same procedure as the microinjections. The dye was localized within the NAc in all animals included in the statistics. Two animals (one ShA and one LgA) were removed from the study because of misplaced cannula. Those animals presented no reduction in responding for cocaine after the administration of the antagonist ($p > 0.05$). Within the NAc, the majority of cannulas were placed in the shell subregion (see histology in Fig. 9). In two LgA animals, one of the two cannulas had an angle or was shifted in medio–lateral plane so that it touched the core subregion, and the other cannula was located in the medial shell. Those animals did present reductions in breakpoints for cocaine.

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Discussion

The following findings provide evidence that eCB system neuroplasticity contributes to the increased motivation for cocaine intake under extended-access conditions: (1) blockade of CB1 receptors by SR141716A systemic administration decreases the breakpoint for cocaine dose-dependently in LgA rats, whereas only the highest dose of the antagonist has an effect in the ShA group; (2) NAc shell eCB tone is maintained by LgA rats during

cocaine self-administration, whereas decreases in eCB levels are observed in ShA rats; (3) pCB1 and CB1 receptors are upregulated in the NAc and amygdala of LgA rats; (4) intra-NAc SR141716A infusions reduce cocaine breakpoints by LgA rats but not ShA rats.

In agreement with previous reports, our LgA rats showed escalated cocaine intake and higher cocaine breakpoints versus the ShA rats, as an index of compulsivity and high motivation to seek cocaine associated with cocaine addiction (Koob, 2008; Wee et al., 2008). We observed enhanced sensitivity to CB1 receptor blockade in LgA rats, suggesting that eCB–CB1 signaling is increased in extended-access animals. Furthermore, SR141716A suppressed responding comparably in LgA animals regardless of whether they were high or low responders for cocaine and reduced cocaine intake by LgA rats but not ShA rats that have similar amount of cocaine in the system during testing, which supports the finding that SR141716A was acting independently of response rate and amount of cocaine in the animals' system and was more potent in LgA rats.

Our present *in vivo* microdialysis study shows that accumbal AEA levels were decreased in ShA rats during cocaine self-administration, whereas this effect was absent in LgA rats. We also did not observe cocaine-related changes in eCB levels after high doses of cocaine in previous studies (Caillé et al., 2007), where animals self-administered cocaine during 90 min dialysis session at high doses (~15 mg/kg) similar to LgA animals of this study (~20 mg/kg vs ~5 mg/kg in ShA rats). Although the relative effect of the amount of cocaine intake on dialysate eCB levels requires further characterization, our present results highlight a potentially important difference in cocaine-induced alterations in NAc eCB levels between LgA and ShA animals. Although baseline AEA levels appear to be lower in the LgA group, the lack of cocaine-induced deficits in interstitial AEA levels in LgA rats suggests maintenance of eCB tone in these animals during cocaine intake that contrasts with a likely reduction in CB1 signaling during cocaine intake by ShA rats. Interestingly, resistance to cocaine-induced decreases in eCB levels apparently is insufficient to observe an effect of intra-NAc SR141716A, according to our previous studies (Caillé et al., 2007). Clearly, some differences between our previous and present studies, such as session duration history and ratio schedules, likely contribute to the differential effects of SR141716A. Of equal or perhaps greater importance are the observations of upregulated pCB1 and CB1 receptor levels in the NAc of LgA rats. The combined observation of relatively greater accumbal AEA levels during cocaine self-administration with increased CB1 receptor protein in this region of LgA versus ShA rats is consistent with enhanced eCB signaling in LgA animals and the relatively greater sensitivity of these animals to CB1 antagonist-induced reductions in the motivational effects of cocaine.

The influence of CB1 receptor phosphorylation on eCB signaling is not well characterized. Recent evidence suggests that phosphorylation of the CB1 receptor distal carboxy terminus promotes internalization of agonist-activated full-length receptors (Daigle et al., 2008) similar to trafficking of delta opioid receptors (Whistler et al., 2001). Thus, it is conceivable that increased pCB1 reflects a compensatory response aimed at reducing CB1-mediated signaling as a consequence of a relative overactivation of the system (Garcia et al., 1998), although it is presently not possible to make strong conclusions in this regard. It is interesting, however, that others noted a correlation between increased CB1 receptor expression and increased pCB1 levels within distinct brain regions (Díaz-Asensio et al., 2008). Finally, demonstration that intra-NAc shell SR141716A infusions reduce cocaine breakpoints selectively in LgA rats supports the hypothesis that the NAc shell is a brain structure where eCB–CB1 signaling may participate in the compulsive and motivational aspects of cocaine addiction (Pontieri et al., 1995). However, we do not exclude an implication of the core subregion in this effect, since we found similar changes in CB1 and pCB1 proteins in both subregions. It is possible that several brain regions (i.e., shell, core, and/or amygdala) act together in the effect observed by SR141716A, since the systemic administration produced stronger reductions in cocaine breakpoints than the intra-NAc shell CB1 antagonist infusions.

CB1 receptors appear to play a role in the determination of the hedonic value and sensitivity to the rewarding properties of natural (Sanchís-Segura et al., 2004; Maccioni et al., 2008) and cocaine reinforcement (Deroche-Gamonet et al., 2001; Xi et al., 2008). Thus, it is possible that the increased motivation to obtain drug (as reflected by the increased effort under PR schedules) is particularly sensitive to SR141716A. This may explain why our LgA rats, which have enhanced motivation to seek cocaine hypothesized to be attributable to changes in hedonic set point (Ahmed et al., 2002), were much more sensitive to the effects of the CB1 antagonist. This hypothesis is consistent with previous reports, where CB1 antagonism was ineffective modulating limited-effort cocaine self-administration under FR schedules (Fattore et al., 1999; Tanda et al., 2000; De Vries et al., 2001; Lesscher et al., 2005; Soria et al., 2005; Caillé and Parsons, 2006; Caillé et al., 2007; Xi et al., 2008) but effective under PR schedules (Soria et al., 2005; Xi et al., 2008). Recently, Xi et al. (2008) showed that high doses of systemic SR141716A were ineffective modulating PR cocaine self-administration in limited-access rats, but 3.0 mg/kg of the potent CB1 antagonist AM251 reduced cocaine responding. We found that SR141716A clearly reduces cocaine breakpoints in LgA rats, even at low doses such as 0.3 mg/kg, which make SR effects more selective for cocaine extended-access rats. However, we also observed a decrease in PR responding in the ShA group with the highest dose of SR, in contrast to Xi et al. (2008). The reasons for the discrepancy between studies are not clear. The fact that intra-accumbal injections of SR141716A in the present study decreased cocaine breakpoints only in LgA rats and not in ShA rats at the doses tested strengthens the conclusion that SR141716A acts selectively for cocaine extended-access animals. However, one cannot exclude the possibility that relatively high doses of SR141716A (this study) or high selective CB1-antagonist (Xi et al., 2008) may also decrease cocaine-maintained behavior by ShA rats.

The neuropharmacological mechanism by which CB1 receptors could modulate motivational aspects of increased cocaine intake is unknown. Studies suggest potential interactions of CB1 receptor activation with the dopamine and/or glutamate systems. Cocaine addiction is associated with decreases in NAc basal dopamine levels (Weiss et al., 1992) and striatal D_2 receptors (Volkow et al., 1999; Nader et al., 2006; Dalley et al., 2007). A compromised postsynaptic D_2-like receptor function in LgA animals may contribute to an upregulated cAMP–PKA signaling in the NAc, which is known to underlie tolerance to cocaine reinforcement (Self et al., 1998; but see Lynch and Taylor, 2005) and is also a requirement for AEA formation in neurons (Cadas et al., 1996). This hypothesis may explain why the LgA rats, which have compromised D_1/D_2 function (Ahmed and Koob, 2004), also present a maintained AEA tone compared with ShA animals (this study). Nevertheless, other reports indicate that, behaviorally, cocaine addiction increased D_2 receptor function or sensitization (De Vries et al., 2002; Edwards et al., 2007), and, interestingly, striatal D_2 activation may be also responsible for AEA release (Giuffrida et al., 1999; Beltramo et al., 2000; Centonze et al., 2004). Alternatively, a maintained AEA tone in LgA rats could be explained by an alteration in the mechanisms of eCB degradation and not in the biosynthesis, as reported for ethanol preference (Hansson et al., 2007).

The CB1 signaling upregulation proposed in this study and the reduced glutamatergic activity observed in escalated animals (Allen et al., 2007) suggest that a retrograde eCB signaling in LgA animals may inhibit glutamate transmission via activation of presynaptic CB1 receptors located in NAc glutamate terminals (Gerdeman et al., 2002; Robbe et al., 2002). Thus, CB1 antagonists could neutralize a CB1-mediated inhibition of glutamate input (Xi et al., 2006) onto AMPA receptors in medium spiny neurons, normalizing the cocaine-evoked NAc synaptic plasticity that underlies the addictive behavior (Bachtell et al., 2008; Conrad et al., 2008). Alternatively, since dopaminergic and eCB systems exert mutual control on each other (van der Stelt and Di Marzo, 2003), it is also conceivable that the dopamine function impairment in LgA rats (Ahmed and Koob, 2004) may be the result of enhanced chronic CB1 neurotransmission, because of an eCB inhibitory feedback mechanism countering dopamine function (Giuffrida et al., 1999; Beltramo et al., 2000).

Together, our results suggest that eCB system neuroplasticity in the NAc may contribute to the motivational drive for cocaine associated with uncontrolled psychostimulant use and, therefore, may be a target for cocaine addiction pharmacotherapies.

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Footnotes

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Several derivatives of cannabinol and the 1,1-dimethylheptyl homolog (DMH)

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Figure1. Compound 4a antagonizes the capacity of the cannabinoid

Cannabinol Derivatives: Binding to Cannabinoid Receptors and Inhibition of Adenylylcyclase

Several derivatives of cannabinol and the 1,1-dimethylheptyl homolog (DMH) of cannabinol were prepared and assayed for binding to the brain and the peripheral cannabinoid receptors(CB 1 and CB 2), as well as for activation of CB 1 -and CB 2 -mediated inhibition of adenylylcyclase. The DMH derivatives were much more potent than the pentyl (i.e., cannabinol) derivatives.11-Hydroxycannabinol (4a) was found to bind potently to both CB 1 and CB 2 (Ki values of 38.0 (7.2 and 26.6 (5.5 nM, respectively) and to inhibit CB 1 -mediated adenylylcyclase with an EC 50 of 58.1 (6.2 nM

but to cause only 20% inhibition of CB 2 -mediated adenylylcyclase at 10 μ M. It behaves as a specific, though not potent, CB 2 antagonist. 11-Hydroxycannabinol-DMH(4b) is a very potent agonist for both CB 1 and CB 2 (Ki values of 100 (50 and 200 (40 pM;EC 50 of adenylylcyclase inhibition 56.2 (4.2 and 207.5 (27.8 pM, respectively).
Chart 1

Introduction
.Two cannabinoid receptors have been described to date. Both are proteins with seven transmembrane-spanning domains. These receptors were originally found in rat brain and spleen, respectively, and are generally known as the central cannabinoid receptor, CB 1, and the peripheral cannabinoid receptor, CB 2. 1,2 CB 1 is mainly expressed in the central nervous system(CNS). It is also found, to a lesser extent, outside the CNS, in numerous other tissues such as vas deferens, adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, thymus, and tonsils. 3,4 The CB 2 gene is not expressed in the brain, being found mostly in the immune system. In certain tissues, such as spleen, the mRNA content of CB 2 is particularly high. In blood cell subpopulations, it is found particularly in B-cells. 3,4 It has been suggested that "cannabinoids may exert specific receptor-mediated action on the immune system through the CB 2 receptor". 4 While a considerable amount of work has been done on structure - activity relationships (SAR) as regards binding to CB 1, 5 - 7 very little is known on binding to CB 2. 8 In the original paper on the identification of CB 2 ,Munro et al. 9 showed that cannabinol (CBN) (1a), which only binds feebly to CB 1, much less so than Δ 9 -tetrahydrocannabinol (Δ 9 -THC) (2a), binds to CB 2 at the samelevel of potency as Δ 9 -THC. The low binding to CB 1 is compatible with the low THC-type in vivo activity recorded for CBN when compared to Δ 9 -THC. 10 The binding of CBN to CB 2, with a potency equivalent to that of Δ 9 -THC, suggests that cannabinol derivatives could serve as a novel starting point for SAR analysisin the cannabinoid series and could possibly lead toselectivity as regards binding to the two cannabinoid receptors. We present here data on several cannabinol derivatives which have been tested for binding to. CB 1 and CB 2. Both CB 1 and CB 2 binding assays were performed with transfected cells. 11 - 13 For comparison purposes, the derivatives were also assayed for CB 1 binding to membranes, as previously described. 14 Various laboratories, including our own, have established that the signal transduction pathway utilized by the two cannabinoid receptors proceeds through pertussis toxin-sensitive G proteins and inhibition of adenylylcyclase activity. 2 We recently showed that Δ 9 -THC binds to both receptors with similar affinity; however, in contrast to its capacity to serve as an agonist for the CB 1 receptor and to inhibit CB 1 -mediated adenylylcyclase, Δ 9 -THC was only able to induce a very slight inhibition of adenylylcyclase at the CB 2 receptor. 12 *

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Cannabinol Derivatives
Scheme 1

Morever Δ 9-THC antagonizes the agonist induced inhibition of adenylylcyclase mediated by CB 2. Therefore,we concluded that Δ 9-THC constitutes a weak antagonist for the CB 2 receptor. 12 It was thus of interest to find out whether CBN derivatives could selectively activate one of the cannabinoid receptors. Therefore, all compounds were tested for CB 1 -as well as CB 2 -mediated inhibition of adenylylcyclase. The present paper is apparently the first one which examines the SAR of cannabinol derivatives for binding to the cannabinoid receptors as well as for inhibition of adenylylcyclase.

Chemistry
Cannabinol (1a), Δ 9 -THC (2a), and cannabidiol (3a) were extracted from hashish 15,16 The 1,1-dimethylhep-tyl (DMH) homolog (2c) (at position 3) of Δ 9 -THC was prepared by Lewis acid-cata lyzed ring cyclization of the DMH homolog of cannabidiol (3b), 17 as previously described for the parallel conversion of cannabidiol (3a) into Δ 9 -THC (2a). 16 The DMH homolog of cannabinol (1b) was prepared by dehydrogenation with sulfur at 240 °C of 2d (synthesized by acetylation of 2c), leading to 1c, followed by removal of the acetate group with ethanolic sodium hydroxide. The isomeric 1,2-dimeth-ylheptyl homolog of CBN has previously been prepared and shown to be a potent cannabimimetic. 18 11-Hy-droxycannabinol (4a) was prepared, as previously described, by selenium dioxide oxidation and dehydrogenation of Δ 9 -THC acetate (2b) followed by removal of the ester grouping. 19 11-Hydroxy- Δ 8 -THC (5c) and its DMH homolog (5a) were prepared as previously described. 19,20 Dehydrogenation of the DMH homolog of11-hydroxy- Δ 8 -THC diacetate (5b) 20 with sulfur at 240°C led to the DMH homolog of 11-hydroxycannabinol diacetate (4c), which was converted to the diol 4b. The same procedure was followed for the conversion of Δ 8 -THC-7-oic acid (6c) 21 into cannabinol-7-oic acid

(7a) 22 and of the DMH homolog of (-) - Δ 8 -THC-11-oic acid (6a) 23 into the DMH homolog of cannabinol-11-oic acid (7c). The lactones 8a, b were prepared following aroute reported by the groups of Adams and Todd about 50 years ago. 24 Von Pechmann condensation of eitherolivetol (9a) or 3-(1,1-dimethylheptyl) resorcinol (9b) with ethyl 5-methyl-1-oxocyclohexane-2 carboxylate (10) led to the known tetrahydrodibenzopyrone 11a or 11b , respectively. 24 Dehydrogenation of 11a or 11b asdescribed above gave 8a 24 or 8b, respectively.

Biological Results
All compounds were tested for binding to CB 1 using African green monkey kidney (COS-7) cells transfected with the cDNA of rat CB 112,25 as well as rat brain synaptosomal membrane preparations. 14,26 Binding to CB 2 was performed using transfected COS-7 and Chi-nese hamster ovary (CHO) cells transfected with the cDNA of human CB 2. 9,11,12 The Ki values were deter-mined by displacement of [3 H] HU-243, a probe for both cannabinoid receptors. 26,27 For consistency, the results discussed below are those from transfected cells except when specifically indicated. All compounds were also assayed for inhibition of CB 1-and CB 2-mediated ade-nylylcyclase. 11,12 The results are summarized in Table1.First, CBN (1a) was compared to Δ 9-THC (2a). Underour experimental conditions, CBN was about 2-4 timesless potent on binding to either CB 1 or CB 2, in contrastto the data reported by Munro et al., 9 who found that CBN and Δ 9-THC are equipotent on CB 2. These differ-ences in recorded potencies are not unexpected, asbinding data are very sensitive to experimental condi-tions. The CB 1-mediated adenylylcyclase inhibitiondata seem to be closer to in vivo observations, where,as mentioned above, Δ 9-THC is considerably morepotent than CBN. We found that CBN is about 13 timesless potent than Δ 9-THC. With CB 2 we see a different profile. While the EC 50 of CBN inhibition of CB 2-mediated cyclase is 261.2 ± 46.4 nM, Δ 9-THC causesonly 21% inhibition at 1 μM. We have previously shownthat Δ 9-THC is a weak agonist for CB 2 and can actuallyantagonize the CB 2-mediated inhibition of adenylylcy-clase caused by more potent agonists. 12 The next derivative to be examined was the DMH homolog of CBN (1b). We observed essentially nodifference between its binding affinity to CB 1 or CB 2. On both receptors, 1b was about 100 times more potent than CBN. It was also 100 times more potent than CBN when tested for binding to rat brain membranes. This compound also potently inhibited adenylylcyclase via both CB 1 and CB 2: the Ki of inhibition by 1b of CB 1 -mediated cyclase was ca. 660-fold lower, and the CB 2-mediated cyclase Ki was ca. 300-fold lower than that of CBN. This result demonstrates that the exchange ofthe pentyl group with the DMH group (at position 3) dramatically increases the affinity of the ligand to bothCB 1 and CB 2 and makes it a much more potent agonist.A similar result was obtained with other cannabinol derivatives (see below). Neither CBN (1a) nor its DMH homolog 1b express any particular selectivity with regard to the inhibition of adenylyl cylase via the two receptors. However, a major metabolite of CBN, namely,11-hydroxy-CBN (4a), 19,22,28 had a different profile. It binds to both receptors with Ki values ca. 5-fold lowerthan the Ki values recorded for CBN. However, while

Table1.
Binding of Various Cannabinoids to Cannabinoid Receptors and Inhibition of Adenylylcyclase a,b
compound
brain synaptosomal binding, (CB 1 (Ki, nM)
binding (Ki, nM)
COS CB 1 and CB 2
CB 1
CB 2
adenylylcyclase (EC 50, nM)
CB 1
CB 2
compound
CBN (1a)
brain synaptosomal binding, (CB 1 (Ki, nM)
392.2 ± 53. 5
COS
CB 1 and CB 2 binding (Ki, nM)
CB 1
211.2 ± 35.0
CB 2
126.4 ± 26.0
adenylylcyclase (EC 50, nM)
CB 1
120.0 ± 32.1
CB 2
261.2 ± 46.4
compound
CBN-DMH (1b)
brain synaptosomal binding, CB 1 (K1, nM)
3.3 ± 0.2
COS CB 1 and CB 2 binding (Ki, nM)
CB 1
2.0 ± 0.3
CB 2
1.5 ± 0.5
adenylylcyclase (EC 50, nM)
CB 1
0.18 ± 0.03
CB 2
0.79 ± 0.021
compound
Δ 9 THC (2a)
brain synaptosomal binding, CB 1 (K1, nM)
66.5 ± 5.8
COS CB 1 and CB 2 binding (Ki, nM)
CB 1
80.3 ± 22.2
CB2 32.2 ± 6.7
adenylylcyclase (EC 50, nM)
CB 1
11.0 ± 2.1
CB 2

21% inhib at 1 μM
compound
Δ 9 - THC-DMH (2c)
brain synaptosomal binding, CB 1 (K1, nM)
2.6 ± 0.3
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
0.241 ± 0.05
CB 2
0.199 ± 0.05
adenylylcyclase (EC 50, nM)
CB 1
0.58 ± 0.12
CB 2
1.01 ± 0.15
compound
11-OH-CBN (4a)
brain synaptosomal binding, CB 1 (K1, nM)
188.9 ± 38.3
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
38.0 ± 7.2
CB 2
26.6 ± 5.5
adenylylcyclase (EC 50, nM)
CB 1
58.1 ± 6.2
CB 2
20% inhib at 10 μM
compound
11-OH-CBN-DMH (4b)
brain synaptosomal binding, CB 1 (K1, nM)
1.8 ± 0.3
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
0.1 ± 0.05
CB 2
0.2 ± 0.04
adenylylcyclase (EC 50, nM)
CB 1
0.056 ± 0.004
CB 2
0.208 ± 0.028
compound
11-OH-Δ 8-THC (5c)
brain synaptosomal binding, CB 1 (K1, nM)
33.4 ± 3.8
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
52.8 ± 2.9
CB 2
7.4 ± 2.5
adenylylcyclase (EC 50, nM)
CB 1
39.9 ± 8.9
CB 2
19% inhib at 1 μM
compound
11-0H-Δ 8- THC-DMH (5a)
brain synaptosomal binding, CB 1 (K1, nM)
0.19 ± 0.01
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
0.1 ± 0.02
0.17 ± 0.01
CB 2
adenylylcyclase (EC 50, nM)
CB 1
0.035 ± 0.005
CB 2
0.078 ± 0.009
compound
Δ 8-THC-11-oic acid-DMH (6a)
brain synaptosomal binding, CB 1 (K1, nM)

480.6 ± 40.8
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
32.3 ± 3.7
CB 2
170.5 ± 7.8
adenylylcyclase (EC 50, nM)
CB 1
927.0 ± 39.6
CB 2
116.2 ± 74.7
compound
Δ 8-THC-11-oic acid-DMH
brain synaptosomal binding, CB 1 (K1, nM)
>1 0000
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
11% in hib at 1 μM
CB2
9% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
no inhib at 1 μM
CB 2
27% inhib at 1 μM
compound
acetate (6b)
compound
CBN-11oic- acid- (7a)
brain synaptosomal binding, CB 1 (K1, nM)
602.4 ± 15.8
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
<2 % inhib at 1 μM
CB 2
23% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
10% inhib at 1 μM
CB2
6% inhib at 1 μM
compound
CBN-11-oic acid acetate (7b)
brain synaptosomal binding, CB 1 (K1, nM)
>1 0000
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
35% inhib at 1 μM
CB 2
19% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
3 inhib at 1 μM
CB 2
2% inhib at 1 μM
compound
CBN-DMH-11-oic acid (7c)
brain synaptosomal binding, CB 1 (K1, nM)
6.1 ± 0.7
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
6.1 ± 1.1
CB 2
4.8 ± 1.9
adenylylcyclase (EC 50, nM)
CB 1
2.4 ± 0.2
CB 2
5.4 ± 0.3
compound
CBN-DMH-11-oic acid
brain synaptosomal binding, CB 1 (K1, nM)
556.1
COS CB 1 and CB 2

binding (Ki, nM)
CB 1
49.8 ± 11.5
CB 2
17.3 ± 7.4
adenylylcyclase (EC 50, nM)
CB 1
13.3 ± 3.4
CB 2
39.3 ± 6.1
compound
acetate (7d)
compound
lactone 8a
brain synaptosomal binding, CB 1 (K1, nM)
>1 0000
COS CB 1 and CB 2
binding (Ki, nM)
14% inhib at 1 μM
adenylylcyclase (EC 50, nM)
CB 1
7% inhib at 1 μM
CB 2
no inhib at 1 μM
compound
lactone 8a
brain synaptosomal binding, CB 1 (K1, nM)
72.5 ± 3.6
COS CB 1 and CB 2
binding (Ki, nM)
CB 1
32.7 ± 4.9
CB 2
17.3 ± 5.6
adenylylcyclase (EC 50, nM)
CB 1
9.6 ± 0.6
CB 2
4.6 ± 0.3
a For details of procedures, see Experimental Section. b For structures, see text.

Figure1. Compound 4a antagonizes the capacity of the cannabinoid agonists 7c (A) and 5a (B) to inhibit the forskolin-stimulate adenylylcyclase activity in CHO cells stably transfected with cDNA of human CB 2. Compounds 4a, 7c, and 5a
were added at the indicated concentrations; 100% represents the amount of cAMP in the absence of cannabinoids. Each point is the mean ± standard deviation of three experiments performed in triplicate.

the EC 50 for the CB 1 -mediated inhibition of cyclase was58.1 ± 6.2 nM, the inhibition of the CB 2 -mediatedcyclase was negligible: 20%inhibition at 10 μM. This result indicates that 4a could serve as a function alantagonist of CB 2 as it binds, but does not activate, CB 2 .Indeed, it reduces the CB 2 -mediated adenylylcyclase inhibitory activity of the potent agonists 5a (HU-210) 5,8b,20 and 7c (Figure 1). However while the adenylylcyclase inhibition by 7c is very strongly reduced by 4a, that of 5a is considerably less. This difference may well be due to the higher inhibitory potency of 5a ,compared to 7c. It should be pointed out that 4a, a major metabolite of CBN, in its differential activity between CB 1 and CB 2, resembles Δ 9-THC

rather than CBN (see above): 12 both Δ 9 -THC and 4a bind to CB 1 and CB 2 and inhibit CB 1 -mediated cyclase in the nanomolarrange but cause only negligible inhibition of CB 2-mediated cyclase up to micromolar concentrations.The DMH homolog of 11-hydroxycannabinol (4b) is the most potent cannabinoid in the present series. It binds to CB 1 and CB 2 with K I values of 100± 50 and 200 ± 40 pM, respectively. Compound 4b also inhibits adenylylcyclase via both CB 1 and CB 2, at very low
concentrations: 56.2 ± 4.2 and 207.5 ± 27.8 pM, respectively. Both the binding constants and EC 50 values for CB 1 and CB 2 are comparable to the values observed for HU-210 (5a). Cannabinol-11-oic acid (7a), 22 as well as its acetate (7b), were essentially inactive on binding to either CB 1 or CB 2, as well as in CB 1- or CB 2-mediated cyclase inhibition. However, the DMH homologs, compounds 7c, d, bind to both CB 1 and CB 2 and inhibit adenylyl-cyclase via the two receptors with high potency. The non acetylated derivative 7c binds with Ki values of 6.1 ± 1.1 and 4.8 ± 1.9 nM to CB 1 and CB 2, respectively, and inhibits cyclase CB 1-and CB 2-mediated activity with EC 50 values of 2.4 ± 0.2 and 5.4 ± 0.3 nM, respectively. These results were unexpected as THC-type 11-oic cannabinoid acids (including DMH ho-mologs) such as 6a, c have not been reported to cause psychotropic effects, 10,21,23 which are presumably mediated by CB 1.The acetate 7d is also active in all theseassays, although its activity is 4-7 times lower than the nonacetylated acid (see Table 1). This differencewas even larger (90 times) when the acetate 7d was compared to the nonacetylated acid 7c with regards to
Cannabinol Derivatives

binding to CB 1 on synaptosomal membranes. It is not clear whether the weaker activity of the acetate is due to its own lower intrinsic activity or to a certain amount of non acetylated 7c formed by hydrolysis during the assays. In view of these results (see Discussion), we looked again into the activity of the DMH homolog of Δ 8 -THC-7-oic acid (6a). 23 This compound, synthesized in ourlaboratory several years ago, was found then to reduce pawedema and leukocyte adhesion to culture dishesand was considered to be antiinflammatory. It did notcause catalepsy in mice at doses up to 1 mg/kg. 23 Wenow find that 6a binds to CB 1 with a rather low Ki of 32.3 ± 3.7 nM but is only a relatively weak inhibitor ofCB 1-mediated cyclase (EC 50 ± 927.0 ± 39.6 nM). Itbinds to the CB 2 receptor with a Ki of 170.5 ± 7.8 nM and inhibits CB 2-mediated cyclase (EC 50 ± 116.2 ± 74.7nM). This material thus affects signaling via CB 2 moreeffectively than via CB 1. The last series to be examined were cannabinol derivatives in which the pyran ring was modified; instead of the two methyl groups on ring B, a ketonewas introduced, thus forming a lactone. Compound 8a (with a pentyl side chain) 24 did not bind to either CB 1 or CB 2 and did not inhibit adenylylcyclase, while theDMH derivative 8b was highly potent, binding to both CB 1 and CB 2 with Ki values of 32.7 ± 4.9 and 17.3 ± 5.6 nM, respectively. It inhibited both CB 1 - and CB 2-mediated adenylylcyclase with EC 50 values of 9.6 ± 0.6 and 4.6 ±
0.3 nM, respectively.

Discussion
For reasons discussed in the Introduction, we assumed that CBN derivatives may show differential binding for CB 1 compared to CB 2 .The results obtained did not fulfill these expectations. Compounds binding poorly to CB 1 (such as 7a, 8a) had the same profile with CB 2, and this was the case also for the more potent compounds (such as 1b,4a,b,7c,d,8b). However, our results proved to be of interest in another direction: we found that binding to CB 2 (see compound 4a) does not necessarily imply a parallel level of inhibition of CB 2-mediated adenylylcyclase. As mentioned above, the major CBN metabolite, 11-hydroxy-CBN (4a), binds well to both CB 1 and CB 2 (Ki) 38 (7.2 and 26.6 (5.5 nM, respectively). However, while 4a inhibits adenylylcyclase strongly via CB 1 (EC 50) 58.1 (6.2 nM), its inhibition of adenylylcyclase via CB 2 is negligible (Table1). Further experiments (see Biological Results and Figure 1) indeed showed that 4a is a weak antagonist to CB 2. Hence, this CBN metabolite may represent a useful target for future research and possibly a tool in CB 2 investigations. As 4a is formed in vivo from CBN, its presence in the body may have physiological consequences associated with CB 2-promoted activities, possibly in the immune system. The replacement of the pentyl group at position 3 with the DMH group increased affinity to CB 1 as well as to CB 2 in all compounds tested. The dramatic increase of pharmacological activity associated with such a structural change was first noted about 50 years ago. 24c,d As indicated above, the DMH homolog of 11-hydroxy-CBN (4b) is the most active compound in the present series as regards binding to CB 1 and CB 2, as well as inhibition of adenylylcyclase (see Table 1). It may serve alongsideHU-210 (5a) as a potent tool in cannabinoid research.

Indeed, 4b shows binding values and cyclase inhibition levels very similar to those of HU-210 (5a) (see Table1).
Oxidation of the 11-CH 3 group in Δ 9 -THC (2a) or Δ 8-THC (12) to a carboxyl group forming THC-7-oic acids (13 or 6c, respectively) leads to inactivation, as seen inbehavioral assays. 10,21 Indeed, this route is the major

inactivation pathway of THC metabolism. The DMH homolog of Δ 8-THC, 11-oic acid 6a, has also beenreported to lack THC-type activity, 23 although thereported test range was limited. Unexpectedly, we now find that although 6a binds to both CB 1 and CB 2 in the 30-170 nM range, the EC 50 of adenylylcyclase inhibi-tion via CB 1 is in the range of 927 ± 39.6 nM. The CB 2 -mediated adenylylcyclase is in the intermediate range(116.2 ± 74.7 nM), i.e., a certain separation betweenCB 1- and CB 2-mediated activation is noted. In theCBN-11-oic acid series, we observed a different profile:while in the pentyl series (7a,b), as expected, werecorded no binding or inhibition of cyclase, with DMH-CBN-11-oic acid (7c), we found potent binding to bothCB 1 and CB 2 and cyclase inhibition (see Table 1).Compounds 6a and 7c differ in the conformation of ringA: a planar aromatic ring in 7c versus a half-chair onein 6a . This conformational difference may represent the molecular basis for the different activities of the two cannabinoids.A comparison of the binding values of 6a with those of its CB 1- and CB 2 -mediated cyclase inhibition (see Table 1) may explain some of the previously reportedproperties of 6a. The relatively high levels of 6a needed to inhibit adenylylcyclase via CB 1 may explain the absence of catalepsy (within the limited dose range tested), in spite of its considerable binding potency,while the ca. 10 times lower levels needed to inhibit adenylyl cylcase via CB 2 may be the basis of its reported antiinflammatory activity. These observations mayserve to open new leads toward the development of antiinflammatory agents in which wider separations of activity can be achieved than these reported now. As mentioned above, all compounds presented inTable 1 were tested for binding both in transfected cellsand in rat brain synaptosomal preparations. While the general potency trend is comparable in the two assays, some individual differences are striking. Compounds 6b,7b, and 8a are inactive in both assays; 7a is inactivein transfected cells and poorly active in the membraneassay. The highly potent 1b,5a, and 7c have the same profile in both assays. However 11-hydroxy CBN-DMH (4b) has a Ki of 100.0 ± 50.0 pM in transfected cells and nearly 20 times higher Ki (i.e., lower potency) in brain membranes. In the compounds within the inter-mediate range, the Ki values differ widely: from about2 times (i.e., essentially equipotent) in 1a,2a, and 8b to about 10 times or morein 7d and 6a. The reasonsfor these differences are not clear, but they should be taken into account when binding data in the cannab-inoid series are compared.In summary, a comparison of the binding potency ofseveral cannabinol derivatives to CB 1 and CB 2 with

their capacity to inhibit adenylylcyclase has led to the discovery of a CBN metabolite (11-hydroxy-CBN, 4a) as a specific, though not potent, CB 2 antagonist and toa new very potent agonist for both CB 1 and CB 2 (11-hydroxy-CBN-DMH, 4b).
Experimental Section
Chemistry.

1 H NMR spectra were measured on a Varian VXR-300S spectrophotometer using TMS as the internal standard. All chemical shifts are reported in ppm. Specific rotations were detected with a Perkin-Elmer 141 polarimeter. Melting points (uncorrected) were determined on a Buchi 530 apparatus. Column chromatography was performed with ICN silica gel 60A. Organic solutions were dried over anhydrousmagnesium sulfate. Elemental

analyses were obtained for all new compounds (or their acetates) and were (0.4% of the theoretical values. The analyses were performed at the Elemental Analysis Laboratory of the Hebrew University. Δ 9-Tetrahydrocannabinol-DMH (2c). Boron trifluorideetherate (5.5 mL) was added to cannabidiol-DMH (3b) 17 (5.7g, 15.4 mmol) in dry dichloromethane (150 mL) containing magnesium sulfate (1 g), under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 20 min. A saturated solution of sodium bicarbonate was added until the red color observed during the reaction faded. The reactionmixture was washed with water, separated, dried, and evapo-rated. The oil obtained was chromatographed on a silica gelcolumn. Δ 9-Tetrahydrocannabinol-DMH (2c) (3 g, 53%) waseluted with 4% ether in petroleum ether: 1 H NMR (CDCl 3) δ 6.38, 6.26 (s, 2H), 6.3 (s 1H), 3.16 (d, 1H, J) 11.1 Hz), 2.14(2H), 0.8-1.8 (m); IR (neat) 3300, 2950, 2850, 1620, 1570 cm-1 .Anal. (C 25 H 38 O 2) C,H. Δ 9-THC-DMH (2c) is not stable. At room temperature itrapidly becomes violet; on TLC after 0.5 h, numerous new spotsare observed. Δ 9-Tetrahydrocannabinol-DMH acetate(2d): 1 H NMR(CDCl 3 δ 6.67, 6.50 (s, 2H), 6.0 (s, 1H), 3.2 (d, 1H), 2.28 (s,3H), 2.14 (2H), 1.9-0.8 (m); IR (neat) 2900, 1760, 1620, 1560cm-1. Dehydrogenations of 2d,5b,6b,d, and 11b. The dehy-drogenations were carried out by heating each compound withsulfur at 238-240 °C, under a nitrogen atmosphere, for ca. 4h. Each mixture was extracted with ether and evaporated. The residue was chromatographed on a silica gel column using variable concentrations of ether in petroleum ether as eluent. Compound 2d led to cannabinol-DMH acetate (1c); compound 5b gave 4c; compound 6b gave 7d; compound 11b gave 8b .For yields and spectroscopic data, see below. Cannabinol-DMH acetate (1c): obtained in 24% yield, mp84-86 °C; 1 H NMR (CDCl 3) δ 7.8, 7.25, 7.13 (s, 3H), 6.85 (d,1H, J) = 2.1 Hz), 6.67 (d, 1H, J) 2.1 Hz), 2.36 (s, 3H), 2.32 (s,3H), 1.6-0.8 (m); IR (neat) 2930, 1780, 1620, 1560 cm - 1 . Anal.(C 27 H 36 O 3) C,H. 11-Hydroxycannabinol-DMH acetate(4c): obtained in44%yield from 5b; 1H NMR (CDCl 3) δ 8.00, 7.25 (s, 3H) 6.85(d, 1H, J) 1.5 Hz), 6.68 (d, 1H, J) 1.5 Hz), 5.10 (s, 2H), 2.34(s, 3H), 2.10 (s, 3H), 1.6-0.85 (m); IR (neat) 2930, 1780, 1740,1620, 1540 cm- 1. Cannabinol-11-oic acid acetate (7b): obtained in 22%yield from 6d , mp 149 -151 °C; 1 H NMR (CDCl 3) δ 8.81 (s,1H), 8.02 (d, 1H, J) 8.1 Hz), 7.38 (d, 1H, J) 8.4 Hz), 6.78,6.64 (s, 2H), 2.42 (s, 3H), 1.7 - 0.8 (m); IR (neat) 2960, 1760,1670, 1610, 1570 cm-1. Anal. (C 23 H 26 O 5) C, H. Cannabinol-11-oicacid-DMH acetate(7d): obtained in20% yield from 6b; 1 H NMR (CDCl 3) δ 8.8 (s, 1H), 8.02 (d, 1H, J) 8.1 Hz), 7.38 (d, 1H, J) 8.4 Hz), 6.86, 6.75 (s, 2H), 2.42(s, 3H), 1.7- 0.8 (m); IR (neat) 2960, 1780, 1680, 1620, 1570cm -1. Anal (C 27 H 34 O 5) C,H. Cannabinol-DMH (1b) : Hydrolysis of 1c in ethanolicsodium hydroxide solution gave compound 1b, mp 95-98 °C; 1 H NMR (CDCl 3) δ 8.11, 7.22, 7.08, 6.36, 6.4 (s, 5H), 2.33 (s,3H), 1.5-0.7 (m). 11-Hydroxycannabinol-DMH (4b): Compound 4c (526mg, 1.13 mmol) in dry ether (10 mL) was added to lithiumaluminum hydride (123 mg) in dry ether (8 mL). The mixturewas boiled under reflux for 2 h. The oil obtained after workup

was chromatographed on a silica gel column (60 g). Elution with 20%ether in petroleum ether gave, after crystallization from pentane, compound 4b (340 mg, 78%): mp 128-130 C; 1 H NMR (CDCl 3) δ 8.45, 7.255, 7.253, 6.60, 6.40 (s, 5H), 4.75(s, 2H), 1.65-0.8 (m, 2H). Anal. (C 25 H 34 O 3) C,H. Cannabinol-11-oic acid-DMH (7c). Compound 7d (60mg) was disolved in 0.6 mL of ethanol. A solution of 60 mg of sodium hydroxide in 0.4 mL of water was added undernitrogen. The solution was stirred at room temperature for 30 minutes. The solution was acidified with 10%HCl (5 mL); the mixture was extracted with ether, dried with MgSO 4, and evaporated. The material obtained was separated on apreparative TLC plate (elution with ether: petroleum ether,7:3), leading to 41 mg of 7c, 76%yield: 1 H NMR 9.2 (s, 1H),8.0 (d, 1H), 7.38 (d, 1H), 6.61 (s, 1H), 6.45 (s, 1H), 1.7-0.8(m). Cannabinol-11-oicacid(7a):7b was hydrolyzed in etha-nolic sodium hydroxide solution as described for compound 7c to yield 7a, 82%yield; 1 H NMR δ 9.2 (s, 1H), 8.0 (d, 1H), 7.38(d, 1H), 6.62 (s, 1H), 6.47 (s, 1H), 1.8-0.8 (m). Compound11b. A solution of 3-DMH-resorcinol (9b) (3.9g, 16.5 mmol), ethyl 4-methyl-2-oxocyclohexane carboxylate (10) (4 g, 21.7 mmol), and POCl 3 (3.06 mL) in dry benzene (15 mL)was boiled under reflux, under a nitrogen atmosphere, for 3h. The solution was washed with NaHCO 3 followed by water. After drying and evaporation the oil was chromatographed ona silica gel column. Compound 11b 24c (40%) was eluted with 10% ether in petroleum ether: mp 158-160 °C (from pentane); 1 H NMR (CDCl 3) δ 6.83, 6.63 (s, 2H), 3.4 (dd, 2H), 2.8 (dd,2H), 2.7-0.8 (m); IR (Nujol) 1680, 1610sh, 1580 cm-1; MS M + 356. Compound 8b: dehydrogenation of 11b with sulfur asdescribed above gave compound 8b (72%), mp 184-185 °C(from pentane); 1 H NMR (CDCl 3) δ 8.82 (s, 1H), 8.3 (d, 1H),7.38 (d, 1H), 6.94, 6.72 (s, 2H), 2.54 (s, 3H), 1.6-0.8 (m, 19H).Anal. (C 23 H 28 O 3) C,H. Receptor Binding Assay. a. Binding to Synaptosomal Membranes. Synaptosomal brain preparations were made from whole rat brain as described previously. 26,27 Binding of [3 H] HU-243 (50.4 Ci/mmol) was assayed in triplicate asdescribed. 26,27 In brief, each reaction mixture of 1 mL insiliconized Eppendorf tubes contained 2.4-3.8 μ g of synapto somal membrane protein, 28-48 fmol of [3 H] HU-243, and various concentrations of competing unlabeled cannabinoids. Tubes were incubated at 30 °C for 90 min and centrifuged at13 000 rpm, and the tips of the tubes containing the pelleted membranes were cut and counted for their radioactivity. b. BindingtoTransfectedCOS-7Cells. Two days after transfection (with 5 μ g/100 mm of dish plasmids encoding CB 1 or CB 2) the cells were washed with phosphate-buffered saline, scraped, pelleted, and stored at-80 °C. Cell pellets were homogenized in binding buffer (50 mM Tris-HCl, 5 mM MgCl 2, and 2.5 mM EDTA, pH 7.4), and 50 μ g protein aliquots were assayed for binding of [3 H] HU-243 as described above, except that the final concentration of [3 H] HU-243 was 300 pM. Formore detailed information see refs 11 and 12.Specific binding was defined as the difference between the amount of radioactivity bound to the pelleted membranes in the absence and presence of 50 nM unlabeled HU-243 (for a)or 1 μM unlabeled 5a (HU-210) (for b) and was typically 70-80% of the total bound. The Ki values for the various cannabinoids and related compounds were calculated from the competition data according to the formula: Ki) IC 50 /1 + ([3 H]-HU-243/ K d). 29 The K d values for HU-243 binding were 45 26 and 61 pM 11 for CB 1 and CB 2 , respectively. Cell Cultures. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8%fetal calf serum, 2 mM glutamine, nonessential amino acids, 100 units/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere consisting of 5%CO 2 and 95%air at 37 °C. CHO cells stably transfected with the cDNA of human CB 2 receptor 9 were described earlier. 11 COS-7 cells in 100 mm dishes were transiently transfected 12 with plasmids encoding rat CB 125 or human CB 29 (5 μ g each) and, when indicated, with 2 μ g of the plasmid containing the cDNA of adenylylcyclase type V. 12 Twenty-four hours later, the cells were trypsinized and cultured in 24-well plates. After an additional 24 h, the cells

were assayed for adenylylcyclase activity. Transfection efficiency, determined by transfection with the cDNA for ⓪-ga-lactosidase, was 40-80%. Adenylylcyclase Assay. The assay were performed as described. 11,12,27 Cells cultured in 24-well plates were incu-bated for 3 h with 0.25 mL/well fresh growth medium contain-ing 5μCi/mL [3 H] adenine. This medium was replaced with Dulbecco's modified Eagle's medium containing 20 mM Hepes (pH 7.4), 1 mg/mL fatty acid-free bovine serum albumin, 0.5mM 1 methyl-3-isobutylxanthine, and 0.5 mM RO-20-1724.Cannabinoids and forskolin (1 μM) were added, and the cells were incubated at 37 °C for 10 min. The reaction was terminated with 1 mL of 2.5%perchloric acid containing 0.1mM unlabeled cAMP. Aliquots of 0.9 mL were neutralized with 100 μL of 3.8 M KOH and 0.16 M K 2 CO 3 and applied toa two-step column separation procedure. 12,27 The [3 H] cAMP was eluted into scintillation vials and counted. Background levels (cAMP accumulation in the absence of forskolin) were subtracted from all values and represented less than 10%offorskolin stimulated cAMP accumulation. Statistical Analysis. Data were analyzed using the Stu dent's t-test. Inhibition curves were generated with the Sigma Plot 4.11 program, and the EC 50 values were determined using an equation from the ALLFIT program. 30 Acknowledgment. This work was supported by NIDA grants DA 9789 and DA 8169 (to R.M.) and a grant by the Israel Science Foundation (to Z.V. and R.M.), the Forchheimer Center for Molecular Genetics(to Z.V.), and the Henry and Anne Reich Research Fund for Mental Health (to Z.V.). M.B. was supported by a fellowship from the Israeli Ministry of Science and Technology. Z.V. is the incumbent of the Ruth and Leonard Shimon Chair of Cancer Research. R.M. is the Lionel Jacobson Professor Medicinal Chemistry. The authors thank Drs. T. Bonner (NIMH, NIH, Bethesda, MD), S. Munro (Cambridge, U.K.), and T. Pfeuffer (Dusseldorf, Germany) for their kind donations of plasmids encoding CB 1, CB 2, and adenylylcyclase type V, respectively.

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Abstract

Cannabinoid pharmacology has made important advances in recent years after the discovery of the cannabinoid receptors. These discoveries have added to our understanding of exogenous and endogenous cannabinoid signaling along with exploring the various pathways of their biosynthesis, molecular structure, inactivation, and anatomical distribution of their receptors throughout the body. The endocannabinoid system is involved in immunoregulation and neuroprotection. In this article, we have reviewed the possible mechanisms of the regulation of the immune response by endocannabinoids which include modulation of immune response in different cell types, effect on cytokine network, induction of apoptosis in immune cells and downregulation of innate and adaptive immune response. Studies from our laboratory have suggested that administration of endocannabinoids or use of inhibitors of enzymes that breakdown the endocannabinoids, leads to immunosuppression and recovery from immune-mediated injury to organs such as the liver. Thus, manipulation of endocannabinoids in vivo may constitute a novel treatment modality against inflammatory disorders.

Keywords: Endocannabinoids, Immune response

Go to:

1. Endogenous cannabinoid system

The discovery of cannabinoid receptors occurring naturally throughout the vertebrate body and the availability of highly selective and potent canabimimetics led to the identification of a naturally occurring lipid signaling system termed the endocannabinoid system. Interestingly, the endocannabinoid system dates back very long in the evolution because it exists as an ancient plant signaling system regulating the plant immunity-related genes in response to infection and stress [1]. The stereoselective binding sites for endocannabinoid ligands have been found in invertebrate immunocytes and microglia showing that this system has been conserved throughout evolution from coelenterates to man and is therefore considered as a very successful evolutionary principle [2]. The endocannabinoid family comprises of a family of lipid transmitters serving as natural ligands for the cannabinoid receptors and the enzymes for biosynthesis and degradation of these ligands. The first endocannabinoid discovered was named as N-arachidonoylethanolamide (anandamide, AEA) from the Sanskrit “internal bliss” by Devane et al. [3]. AEA is an amide of arachidonic acid and ethanolamine. Subsequently, 3 years later, another ligand named 2-arachidonoylglycerol (2-AG) was discovered independently by Mechoulam et al. [4] and Sugiura et al. [5], which was found to be in much higher concentration in serum and brain than anandamide.

Mammalian tissues are known to express primarily two types of cannabinoid receptors which are G-protein coupled. These include CB1 receptors, cloned in Tom Bonner’s laboratory in 1990 and CB2 receptors, cloned by Sean Munro in 1993. Both CB1 and CB2 receptors regulate the release of chemical messengers, where CB1 receptors are found mainly on neurons (and in some non-neuronal tissues like pituitary gland, immune cells and reproductive tissues), and CB2 receptors are found primarily on immune cells. Anandamide binds to the brain CB1 with high affinity and mimics the behavioral actions of exogenous cannabinoid, Δ9-tetrahydrocannabinol (Δ9-THC) when injected into rodents. 2-AG has similar affinities for both CB1 and CB2 receptors comparable to those of anandamide but it exhibits higher efficacy than anandamide. Other pharmacological targets for anandamide, other than cannabinoid receptors, have also been described. The most accepted receptor to be activated by anandamide and other synthetic analogs of endocannabinoids (methanamide, arachidonyl-2′-chloroethylamide) is TRPV1 (vanilloid VR1) receptor [6,7]. These findings have triggered an exponential growth in studies describing the endocannabinoid synthesis, their release [8], transport [9] and degradation [10], constituting the new ubiquitous “endogenous cannabinoid system”.

The main pharmacological functions of the endocannabinoid system include neuromodulation, controlling motor functions, cognition, emotional responses, homeostasis and motivation. However, in the periphery, this system is an important modulator of autonomic nervous system, the immune system and microcirculation.

1.1. Biosynthesis of endocannabinoids

Endocannabinoids are not stored in vesicles or cells like neurotransmitters; rather their biosynthesis takes place on demand from lipid precursors present in the cytoplasmic membrane through enzyme activation in response to elevations of intracellular calcium. The immediate precursor to AEA is N-arachidonoyl phosphatidylethanolamine, which is formed from phosphatidylcholine and phosphatidylethanolamine and is converted to anandamide by the action of N-arachidonoyl phosphatidylethanolamine-phospholipase [11]. Synthesis of 2-AG depends on the conversion of 2-arachidonate-containing phosphoinositides to diacylglycerols (DAGs), which are then converted to 2-AG by the action of DAG lipase. Also, like anandamide, 2-AG is thought to be removed from its sites of action by cellular uptake and metabolized intracellularly [12]. Release of endocannabinoids is stimulated in a receptor-dependent manner by neurotransmitters. It is noteworthy that some effects of endogenously released anandamide and 2-AG may be enhanced through an “entourage effect” or ‘ALIA’ mechanism that relies on the corelease of other endogenous fatty acid derivatives as first described by Facci et al. [13]. These derivatives include palmitoylethanolamide (PEA) and oleamide, which can potentiate anandamide, and 2-linoleyl glycerol and 2-palmitoyl glycerol, which can potentiate 2-AG [6].

The endocannabinoids are removed from the site by cellular uptake processes such as simple diffusion, through membrane associated binding proteins or by a transmembrane carrier protein. Inside the tissue, their metabolism is catalyzed by fatty acid amide hydrolase (FAAH) [12,14]. FAAH is found postsynaptically mainly in soma and dendrites of principal neurons and is located on cytosolic surfaces of SER cisternae and mitochondria. In some tissues, however, endocannabinoids also undergo an oxidative catabolism through palmitoylethanolamide-preferring acid amidase (PAA) lipoxygenases, cyclooxygenase-2 and cytochrome P450. Thus, anandamide and 2-AG can be released from both neuronal and non-neuronal cells

whenever the need arises, and they utilize analogous but distinct receptor-dependent pathways regulating the effects of primary messengers, such as neurotransmitters and hormones. In certain disorders such as multiple sclerosis, cancer, intestinal disorders, cardiovascular disorders, pain, Parkinson's disease and excitotoxicity, the tissue concentration of endocannabinoids, cannabinoid receptor density and the cannabinoid receptor coupling efficiency increases resulting in the reduction of symptoms of these disorders. The endocannabinoid system has been shown to be involved in various physiological processes like lipogenesis, inflammation, food intake and nociception.

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2. Endocannabinoid receptors in immune system

Endocannabinoids are believed to control immune functions and play a role in immune homeostasis. Immune cells express both CB1 and CB2 receptors, secrete endocannabinoids and have functional cannabinoid transport and breakdown mechanisms [15,16]. Human peripheral blood immune cells are reported to have different degrees of cannabinoid receptor expression with the following rank order: B cells > NK cells > monocytes > polymorphonuclear neutrophils > CD8 lymphocytes > CD4 lymphocytes [17]. The CB1 receptors are densely expressed in the central nervous system and mediate neurobehavioral effects. The expression levels of CB2 receptors in immune cells are 10–100 times greater than CB1 receptors. Moreover, CB2 receptor mRNA was also detected in the cortex of lymph nodes and the nodular corona of Peyer's patches [18].

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3. Endocannabinoid biosynthesis and signaling in the immune system

There is significant biochemical evidence to suggest that biosynthesis, uptake and degradation of endocannabinoids occur in macrophages and leukocytes [15,16,19]. This finding supports the role of endocannabinoids as local modulators of immune and inflammatory reactions. It was observed that both RBL-2H3 basophil cells and J774 macrophages can biosynthesize AEA and PEA through hydrolysis of corresponding N-acylphosphatidylethanolamines. Both these cell lines could inactivate the two bioactive acylethanolamide by sequestering radiolabelled AEA and PEA from the culture medium. Furthermore, following uptake by basophils, AEA and PEA competed for the same inactivating enzyme which catalyzed their hydrolysis to ethanolamine. Maccarrone et al. demonstrated that LPS down-regulated FAAH expression and increased AEA levels in human peripheral lymphocytes [20,21]. The effect of this endotoxin on FAAH was not mediated by AEA-induced activation of cannabinoid receptors. In fact, the stimulatory action of LPS on AEA levels was considered to be due to the inhibition of FAAH as suggested by the observation that an increase in levels of AEA was also induced by an irreversible FAAH inhibitor. Varga et al. demonstrated that rat platelets and macrophages contained the endogenous cannabinoid, 2-AG, and in vitro exposure to LPS markedly increased 2-AG levels [22]. Thus, these results indicated that immune cells take part in regulating peripheral endocannabinoid system and endocannabinoid homeostasis. It has been shown that human mast cells also take up AEA followed by its hydrolysis by FAAH [21]. Thus, mast cells contribute towards regulation of peripheral endocannabinoid system thereby affecting endocannabinoid mediated effect in inflammation, vascular tone and other immune interactions [21].

The CB1 and CB2 receptors negatively regulate adenylyl cyclase activity through pertussis toxin-sensitive GTP-binding protein. Endocannabinoids have been well known to exhibit this property, which is considered to be an important mechanism of lymphocyte regulation [4,23]. As cAMP signaling cascade has a positive regulatory role in immune cell function, cannabinoid receptor stimulation could antagonize the early events in immune cell activation. Besides, CB1 and CB2 receptors also stimulate mitogen activated protein kinase (MAPK) activity. Endocannabinoids have also been shown to induce MAPK pathway, which is a CB2 receptor-mediated response [24]. Thus, it is now evident that cannabinoid receptor stimulation in the immune system by the endocannabinoids triggers a complex regulation and modulation of cAMP pathways and the cannabinoid receptor stimulation of MAP kinase plays a key role in immune homeostasis and control. The importance of CB2 receptor activation in the immunomodulatory effects of endocannabinoids has been recognized and is supported by anti-inflammatory effects of CB2 receptor activation in many pathological conditions and disparate diseases ranging from inflammatory pain, myocardial infarction, stroke, hepatic I/R injury, gastrointestinal inflammatory disorders [25,26], liver inflammatory disorders [27] and atherosclerosis [28].

The effect of cannabinoids on immune functions appears to be transient which would allow the inhibitory effects to be overcome when the immune system needs to be activated during infections. This is supported by the downregulation of cannabinoid receptor expression when the immune cells are activated. Thus, the transient nature of cannabinoids on the immune system suggests that the side effects of the potential therapy may be minimal. Although the function of cannabinoid receptor on immune cells and the cross-talk between the immune system and endocannabinoids is yet to be fully defined, based on available data, we can hypothesize that the endocannabinoid signaling in lymphoid tissue may provide a tonic control of immune cell activation and therefore limit spontaneous activation of immune cell function. There have been a number of recent studies which have demonstrated that the endocannabinoids have both inhibitory effects and stimulatory impact on the immune system and may be actually important in homeostasis or control of the immune reactions.

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4. Endocannabinoid system in the CNS immunity and inflammation

The inflammatory network of the CNS is an orchestrated collaboration of neuronal and non-neuronal cells, such as T-lymphocytes, oligodendrocytes, astrocytes and microglial cells. In recent years, there have been several in vitro, in vivo and clinical studies suggesting that endocannabinoid system plays an important role in the cellular network of communication in and between the nervous and immune system during neuronal damage and neuroinflammation. During inflammation in the CNS, there is a loss of balance and control, resulting in neuronal damage and neurological diseases [29]. Inflammatory processes are involved in many acute and chronic neurodegenerative autoimmune disorders. 2-AG in brain is produced in response to intracellular Ca2+ and stimulation of glutamate receptors, and is found at 200-fold higher concentrations in brain tissue. 2-AG is produced by microglial cells and astrocytes in response to stimulation of purinergic P2X7 receptors [30]. During injury, the ATP inside neuronal cells flood the extracellular space and thus stimulate purinergic receptors on microglia and astrocytes causing release of endocannabinoids on neuronal damage. This cellular endocannabinoid response is a restricted process involving short and local events affecting only few cells in vicinity.

Synthesis of AEA in the CNS is well regulated, and is released only on demand. Recently, it has been suggested that AEA is released by CNS tissue as a mechanism that controls and limits immune response in healthy and damaged brain [31]. It has also been reported that endocannabinoid-mediated neuroprotection is impaired during experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, as a consequence of high levels of IFN-gamma which disrupts the functionality of purinergic P2X7 receptors [31]. Mice lacking the CB1 receptor develop more severe neuronal damage following EAE induction, supporting a role for endocannabinoids in this inflammatory neurodegeneration [32]. Furthermore, pharmacological interventions aimed at enhancing the levels of the anandamide significantly attenuated the clinical and pathological features of EAE [33,34]. The homozygous 188–189 GG/GG polymorphism in CB2 receptor (substitution of glutamine at amino acid position 63 by arginine) has been shown to be associated with autoimmune disorders [35]. Proliferative response in T-lymphocytes from CB2 188–189 GG/GG homozygotes was observed to be less susceptible to endocannabinoid-induced suppression. Dexanabinol (HU-211), a synthetic nonpsychotropic cannabinoid, significantly reduced the clinical symptoms of EAE and suppressed inflammation [36]. Arvanil, a synthetic capsaicin–anandamide

hybrid has been shown to ameliorate EAE [37]. The neuroprotection provided by endocannabinoids has been considered to be a direct effect through CB1 receptors that are widely distributed in the mammalian brain in neocortex, hippocampus, basal ganglia, cerebellum and brainstem [38].

The existing literature is highly supportive of a beneficial immunomodulatory action mediated by endocannabinoid activation during both MS and EAE [39]. Virtually all immune cells involved in the pathophysiology of MS and EAE express functional cannabinoid receptors [40] and evidence exists that stimulation of these receptors mediates complex inhibitory actions in these cells, accounting, at least in part, for the protective effects of direct and indirect cannabinoid agonists in MS and animal models of this disease [33,34,41]. Recently, higher concentrations of AEA were reported in active lesions than in quiescent lesions in MS patients [31]. It has been observed that AEA and 2-AG, although sharing many pharmacological actions, have differential regulatory mechanisms, and that AEA but not 2-AG may be involved during pathological events [42]. Similar to this finding it was observed that AEA, but not 2-AG, was increased in the CSF of MS patients, indicating that the two endocannabinoids are differentially engaged during CNS inflammatory diseases [43].

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5. Endocannabinoid and immune modulation

Endocannabinoids have important effects on immune functions. They modulate T- and B-lymphocytes proliferation and apoptosis, macrophage-mediated killing of sensitized cells, inflammatory cytokine production, immune cell activation by inflammatory stimuli, chemotaxis and inflammatory cell migration [44,45]. The immunosuppressive effect of endocannabinoids on immune cells is primarily considered to be mediated through CB2 receptors by the inhibition of the cAMP/protein kinaseA (PKA) pathway (by decreasing the expression of cAMP-responsive genes). Endocannabinoids also act at nuclear level, e.g. phosphorylation of IκB-α that enhances the transcription of several apoptotic genes regulated by NF-κB, by peroxisome proliferator-activated receptor gamma (PPAR-γ dependent inhibition of nuclear factor of activated T cells (NF-AT) and interference of cell cycle by activation of p21waf-1/cip-1 and induction of G1/S phase arrest [37].

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6. Lymphocytes and haematopoietic cells

Schwarz et al. [46] demonstrated dose-dependent immunosuppressive effects of AEA on T and B human lymphocyte proliferation. AEA effect on DNA synthesis in T- and B-lymphocytes occurred very rapidly as the exposure of the cells during the final 4 h of culture was sufficient to achieve >40% inhibition. Low doses of AEA caused significant inhibition of lymphocyte proliferation and DNA fragmentation, inducing cell death by apoptosis. 2-AG has been shown to exhibit biological activity in mouse splenocytes by producing strong immunomodulatory activity on mitogen induced lymphocyte proliferation, mixed lymphocyte response, and on antibody forming cell responses to T-cell dependent and T-cell independent antigens [47]. However, this immunoenhancing effect of 2-AG is considered to be a part of rapid degradation of 2-AG into AA which could in turn serve as a precursor for prostaglandin E2, which is a well characterized activator of adenyl cyclase, important for lymphocyte proliferation and antibody forming cell responses.

The antibody producing B cells have been shown to express high levels of CB2 receptors. Role of endocannabinoids has been implicated in migration of B cells along with induction of some cytokines when present in low doses. Several in vivo and in vitro studies have demonstrated the inhibition of antibody formation by endocannabinoids and synthetic cannabinoids at micromolar concentrations [48,49]. This effect was shown to be mediated by cannabinoid receptor and cAMP-dependent signaling elements in vitro as pertussis toxin could block the suppressive effect on antibody formation in splenocyte culture [48]. In part, the effect of endocannabinoids on modulating the serum levels of immunoglobulin is also considered to be due to their effect on Th cell cytokine secretion/polarization rather than the direct effect on B cells themselves. The endocannabinoid, 2-AG, could stimulate the migration of splenocytes in a CB2-dependent manner [50]. In addition, CB2 receptor stimulation may be associated with B cell differentiation [51], suggesting the positive role of endocannabinoids in mobilizing B cells during immune functions. However, it is yet to be explored whether endocannabinoids exhibit a direct effect on B cells or affect indirectly through T cells and macrophages required for B cell activation. Valk et al. demonstrated that AEA stimulated proliferation of haematopoietic cell lines in synergy with other growth stimuli such as GM-CSF, G-CSF and IL-3 [52]. Derocq et al. also showed that AEA potentiated the growth of cytokine dependent haematopoietic cell lines [53]. However, this effect was not reversed by receptor antagonists, thereby providing evidence for CB1 and CB2 receptor-independent effects. Arvanil, a synthetic capsaicin–anandamide hybrid has been shown to downregulate activation of CD4+ T cells [37] and 2-methylarachidonyl-(2′-fluoroethyl)amide(F–Me–AEA), an analog of anandamide was found to inhibit forskolin-stimulated adenylate cyclase activity in splenocytes and thymocytes [54]. It has been suggested that the regulation of PPAR-γ activity is one of the mechanisms by which endocannabinoids influence T cell activity [55].

The effects of endocannabinoids on T cell cytokine production demonstrated that both TH1 and TH2 cytokines can be either inhibited or induced. AEA and PEA were tested on LPS and phytohemagglutin (PHA)-stimulated human peripheral blood mononuclear cells in vitro and on LPS-induced pulmonary inflammation in mice and results showed the downregulation of LPS-induced synthesis of TNF-α, IL-6, IL-8 and PHA-induced IL-4 synthesis at nanomolar concentrations [56]. IFN-γ is an important mediator in delayed-type hypersensitivity reactions. AEA also inhibited PHA-induced IFN-γ synthesis, however, PEA was ineffective thereby indicating the specific cell sensitivity to different molecular species of ethanolamides and pointing towards the importance of fatty acid ethanolamides as possible endogenous autocoids playing multiple down regulatory roles in the complex network of immune responses. 2-AG also caused human monocytes to produce decreased levels of cytokines and adhesion molecules, thereby exhibiting an immunosuppressive response. In phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore-stimulated splenocytes, 2-AG suppressed IFN-γ mRNA expression in a concentration-dependent manner, which was shown to be independent of CB2 receptor and mediated through inhibition of NF-AT nuclear translocation. Th1 type cytokines have been implicated in the pathogenesis of a number of autoimmune disorders such as multiple sclerosis, its animal model (EAE), rheumatoid arthritis and autoimmune hepatitis (AIH). Inhibition of Th1 cytokines and the shift to Th2 type response is considered to provide therapeutic benefit. Endocannabinoids have been shown to cause blockade of Th1 cytokines and increase the expression of Th2 cytokines [57,58], such as IL-4 and IL-10 that are important for humoral immunity and TGF-α which has immunosuppressive properties [59]. On the other hand, cytokines have been shown to influence directly the endocannabinoid system such as by regulation of degrading enzymes: IL-4 or IL-10 stimulated FAAH activity, whereas IL-12 and IFN-gamma reduced FAAH activity and protein expression of FAAH [20]. In activated Jurkat T cells, significant suppression of IL-2 by 2-AG was shown to be induced at concentrations as low as 1 μM, which is within an order of magnitude of the calculated endogenous 2-AG levels detected in human plasma. This suppression was mediated by a COX-2 metabolite of 2-AG [55].

Recently, our lab has reported that both exogenous and endogenous cannabinoids can attenuate concanavalin A (ConA)-induced acute hepatitis, a well established model for autoimmune hepatitis in which liver injury is T cell-mediated [60]. In our studies, exogenously administered anandamide in the hepatitis model led to a decrease in hepatic injury which correlated with decreased activities of liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) levels, and other inflammatory cytokines. We also observed that 12 h after anandamide treatment, in ConA-injected mice, there was a significant decrease in inflammatory cytokines TNF-α, IL-1β, IL-6, IL-9, and IL-17 and in chemokines such as KC, eotaxin and monocyte chemoattractant protein-1. Blocking of CB1 or CB2 receptors reversed the anandamide-mediated suppression of hepatitis thereby

indicating that anandamide was acting in a CB1- and CB2-dependent manner. Anandamide levels are elevated in the absence of FAAH activity [10].Mice which lacked FAAH enzyme (responsible for hydrolysis of anandamide) were found to be resistant to ConA-induced hepatitis and showed less severe liver tissue damage and decreased leukocyte infiltration [60]. Similarly, increased endogenous anandamide levels caused by administering mice with FAAH inhibitors MAFP or URB532, also caused a decrease in hepatic injury with significant decrease in AST levels upon ConA challenge. These findings are exciting and suggest the potential manipulation of endocannabinoids, including the use of FAAH inhibitors in the treatment of AIH.

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7. Macrophages

Macrophages play an important role in both innate and adaptive immunity by mediating their effect through presenting antigen to T cells, phagocytosis of infectious agents and secreting acute phase proteins such as nitric oxide, TNF-α, IL-1 and IL-6. CB1 and CB2 receptors are widely expressed on monocytes/macrophages and microgial cells. AEA has been shown to inhibit macrophage-mediated killing of TNF-sensitive murine L929 fibroblasts [61]. AEA and PEA have also been shown to maximally inhibit LPS-induced expression of pro-inflammatory mediators such as nitric oxide production in murine RAW264.7 macrophages and microglia [62] and these effects were mediated by CB2 receptors. Whereas AEA diminished LPS-induced NO and interleukin-6 production, 2-AG inhibited IL-6 production but increased iNOS-dependent NO production [63]. It has been suggested that the discrepant results of 2-AG could be due to its bioactive metabolites, AA and PEG2. 2-AG enhanced the adhesion of macrophage-like differentiated HL-60 cells to fibronectin and to vascular cell adhesion molecule-1 (VCAM-1) and adhesion of human monocytic leukemia U937 cells and peripheral blood monocytes. This enhanced adhesion has been suggested to be mediated by CB2 receptor, Gi–G0-proteins, and phosphotidylinositol 3-kinase dependent pathways. Decreased accumulation of macrophage-like cells was observed at focal sites of infection in a murine model of Granulomatous Amebic Encephalitis and reduced macrophage chemotaxis by cannabinoids was observed in a murine model of atherosclerosis, suggesting their strong effect on macrophage migration which is mediated by CB2 receptor signaling. In a recent study, patients with coronary artery disease demonstrated the activation of the endocannabinoid system with elevated levels of blood endocannabinoids and increased expression of CB1 receptor in coronary atheroma, particularly in macrophages. CB1 receptor blockade exhibited anti-inflammatory effects on human macrophages, which might provide beneficial effects on atherogenesis [64]. Thus, these properties of endocannabinoids provide a rationale for their use as anti-inflammatory agents.

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8. Mast cells

Mast cells are bone marrow derived, multifunctional immune cells populating connective and mucosal tissue as well as nervous system and are involved in inflammatory reactions. Using mast cell lines it was shown that they express both CB1 and CB2 receptors. Both PEA and AEA bound to CB2 receptors but only PEA could down modulate mast cell activation in vitro and this effect was efficiently antagonized by AEA [13]. Endocannabinoids except PEA and PEA derivatives have also been shown to induce a non-lytic, energy and concentration-dependent histamine release in rat peritoneal mast cells [65]. However, this effect was shown to occur irrespective of the presence of cannabinoid receptors. The effect was partially reduced by pertussis toxin indicating that Gi- and G0-protein were partly responsible for the effect. In addition, where synthetic ligands enhanced anti-IgE mediated histamine release, AEA and PEA treatment showed no effect. In contrast, it has been demonstrated that 2-AG mediated suppression of histamine release from guinea pig mast cells can be reversed by nitric oxide synthase inhibitor or a CB2 receptor antagonist [66]. While analyzing the effect of endocannabinoids on LPS-induced bronchopulmonary inflammation in mice, PEA was shown to diminish the level of TNF-α by 31.5% but with no effect on neutrophil recruitment. AEA, however, did not influence the inflammatory process but TNF-α level and neutrophil recruitment was decreased by 28% and 62%, respectively. Interestingly, AEA levels were found to be upregulated in biopsies from patients with colitis or in patients with diverticular diseases [67]. According to the notion that endocannabinoids are released on demand, such findings suggest their possible role in the restoration of tissue homeostasis in certain pathological conditions. In a recent study it was observed that CB1 and CB2 agonists prevented mast cell-dependent angiogenesis during granuloma formation, thus indicating that cannabinoids can control mast cell activation in a receptor-dependent manner [68].

Although PEA does not bind with high affinity to CB1 (Ki = 23.8 nm) or CB2 (Ki = 13.9 nm) receptors, it still maintains CB-like anti-inflammatory actions in several mast cell-mediated experimental models of inflammation. For example, PEA reduces edema in response to compound 48/80 [69] and carrageen [70]. Recently, a new drug containing PEA has been approved by Food and Drug administration for treatment of dermatitis. A recent pilot study aimed to assess the efficacy and safety of twice daily application of a topical emulsion containing 2% Adelmidrol, a PEA analog, in 20 pediatric patients suffering from atopic dermatitis showed 80% increase in symptom resolution via inhibition of nerve growth factor (NGF) release from mast cells [71].

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9. Dendritic cells (DCs)

DCs play a major role as antigen-presenting cells and in the development of antigen-specific T cell responses. Human dendritic cells have been shown to express both CB1 and CB2 receptors. There have been several reports indicating that the endocannabinoid system plays a critical role in regulating DC growth and maturation [72]. The endogenous cannabinoid system is present in DCs and can be regulated by cell activation [72]. AEA, 2-AG and PEA were observed in lipid extracts from immature dendritic cells. Upon LPS activation, the quantity of 2-AG was increased in DCs. However, there was no increased expression of CB1, CB2 or FAAH observed. Furthermore, 2-AG has been suggested to act as a chemotactic molecule capable of recruiting DCs during innate immune response and in the presence of a TLR agonist, instructs a Th1-shifted adaptive response [73].

DCs are highly sensitive to cannabinoid-induced apoptosis which is considered as a cellular background of immunosuppressive property of endocannabinoids [74]. Studies from our laboratory demonstrated for the first time that both exogenous and endogenous cannabinoids induce apoptosis in DCs [74]. To investigate whether endogenous cannabinoids such as anandamide could induce apoptosis in DCs, various concentrations of anandamide were added to DCs. It was observed that concentrations of 20 μM caused marked apoptosis while lower concentrations were not effective. The lesser efficacy of anandamide as compared to exogenous cannabinoids might be due to rapid hydrolysis of anandamide by endogenous enzymes such as FAAH. To prevent the activity of FAAH, various concentrations of a FAAH inhibitor were added to the culture in the presence or in the absence of anandamide and the induction of apoptosis was determined. These studies revealed that addition of a FAAH inhibitor could increase the induction of apoptosis by anandamide in DCs significantly. Furthermore, anandamide-induced apoptosis was mediated through cannabinoid receptors since addition of selective antagonists of CB1 (SR141716A) or CB2 (SR144528) to the cultures reversed the effects of anandamide.

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10. Natural killer cells and neutrophils

Natural killer cells and neutrophils are involved in host defense against cancer and anti-microbial responses. In vitro studies on human NK cells demonstrated that cannabinoids can inhibit the constitutive expression of chemokines, IL-8, MIP1-α, MIP-1β, RANTES, phorbol ester stimulated TNF-α, GM-CSF and IFN-gamma. Studies have demonstrated that cannabinoids can suppress NK cell function such as cytolytic activity in rats, mice and humans, which is a cannabinoid receptor-dependent process [75–77].

The presence of cannabinoid receptors on neutrophils was first reported in 1993. Dose-dependent non-cytotoxic release of lysosomal enzymes from neutrophils has been shown to be induced by cannabinoid ligands. Recent studies have also shown that AEA failed to inhibit superoxide production in neutrophils although other synthetic cannabinoid treatment exhibited the inhibition property. This effect was shown to be independent of cannabinoid receptors [78].

10.1. Endocannabinoids and cancer

Recent applications of cannabinoids have included their potential use as anti-tumor agents [79,80] which relies on their ability to inhibit tumor angiogenesis [81,82] or directly induce apoptosis or cell cycle arrest in neoplastic cells [83–86]. Studies from our laboratory demonstrated that AEA can induce apoptosis in malignant immune cells [87]. Molt-4 human tumor cells when cultured for 4 h in the presence of various concentrations of anandamide (5, 10, 20, and 40 μM) were found to undergo significant levels of apoptosis as quantified using the TUNEL assay at concentrations of 20 μM or greater. Also, murine EL-4 tumor T cells were found to be more sensitive to AEA as much as exposure to 5 and 10 μM AEA was sufficient to trigger significant levels of apoptosis [87]. Role of the endocannabinoids as potential endogenous tumor growth inhibitors has been suggested in a study where it was observed that levels of both AEA and 2-AG were higher in precancerous polyps than in fully developed carcinoma in colon [88]. Recent in vivo studies proposed that selective targeting of CB2 receptor resulted in colorectal tumor growth inhibition via apoptosis which was mediated through the stimulation of ceramide [89]. In a xenograft model of thyroid cancer, substances that block endocannabinoid degradation increased levels of AEA and 2-AG in tissues and reduced tumor growth [90]. Various attempts have been made to inactivate cannabinoid degrading enzymes thus increasing the local concentration of endocannabinoids at the tumor cell surface leading to anti-tumor effects of CB-receptor signaling in various types of cancer including thyroid, brain and prostate cancers [90–93].

Although majority of the effects of cannabinoids are mediated through CB receptors, AEA has been shown to induce its effects on cancerous cells by interacting with TRPV1 receptor [94,95] or cholesterol rich lipid rafts [96]. Furthermore, it has been reported that signaling pathways are differentially regulated by cannabinoids in normal cells versus cancer cells. In malignancies, such as thyroid cancer, lymphoma, melanoma, pancreas and breast cancer, the levels of cannabinoid receptors are often higher in the tumors when compared to normal cells, resulting in increased sensitivity to cannabinoids in malignant cells [67,83,97–99]. Moreover, many animal studies have reported anti-proliferative and pro-apoptotic effects of cannabinoids on tumor cells but not on normal tissue [79,83,85,97]. Thus the role of the endocannabinoid system in cancer indicates that this system is involved in regulating many of the functions that are essential in cancer development.

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11. Concluding remarks

The endocannabinoids exhibit complex regulatory effects on the immune system. As summarized in Table 1, endocannabinoids are involved in immune regulation by the suppression of cell activation, inhibition of pro-inflammatory cytokine production, NF-κB-dependent apoptosis and modulation of the functions of T helper subsets: Th1 and Th2. Endocannabnoids (AEA and 2-AG) and their congeners (PEA) can thus be considered as potent immunomodulators. The effect of endocannabinoids depends on cell type, their concentration and cellular environment. The effects of endocannabinoids on chemokines, adhesion molecules and expression of costimulatory molecules need further evaluation. To understand the pleotropic effects of endocannabinoids on immune system, future studies on differential cannabinoid receptor signaling using knockout mice and further identification of cannabinoid-like receptors needs to be undertaken in the coming years. It is also critical to know the production and regulation of endocannabinoids by the naïve and activated components of the innate and adaptive immune response. The image of endocannabinoid system now appears to be of a modulatory complex which affects the physiological functions in peripheral tissues and can thus be considered as a potential therapeutic target in the future. While several in vitro studies have delineated the immunomodulatory effects of endocannabinoids, their in-depth functions in vivo need to be addressed. Use of inhibitors of enzymes such as FAAH that breakdown the endocannabinoids, to modulate their levels and consequent regulation of immune response, can provide novel therapeutic modality against inflammatory and autoimmune diseases.

Table 1

Table 1

Effect of endocannabinoids on functions of different immune cells.

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Footnotes

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Endocannabinoid system

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The endocannabinoid system (ECS) is a biological system composed of endocannabinoids, which are endogenous lipid-based retrograde neurotransmitters that bind to cannabinoid receptors, and cannabinoid receptor proteins that are expressed throughout the mammalian central nervous system (including the brain) and peripheral nervous system. The endocannabinoid system is involved in regulating a variety of physiological and cognitive processes including fertility,[1] pregnancy,[2] during pre- and postnatal development,[3] appetite, pain-sensation, mood, and memory, and in mediating the pharmacological effects of cannabis.[4][5] The ECS is also involved in mediating some of the physiological and cognitive effects of voluntary physical exercise in humans and other animals, such as contributing to exercise-induced euphoria as well as modulating locomotor activity and motivational salience for rewards.[6][7][8][9] In humans, the plasma concentration of certain endocannabinoids (i.e., anandamide) have been found to rise during physical activity;[6][7] since endocannabinoids can effectively penetrate the blood–brain barrier, it has been suggested that anandamide, along with other euphoriant neurochemicals, contributes to the development of exercise-induced euphoria in humans, a state colloquially referred to as a runner's high.[6][7]

Two primary endocannabinoid receptors have been identified: CB1, first cloned in 1990; and CB2, cloned in 1993. CB1 receptors are found predominantly in the brain and nervous system, as well as in peripheral organs and tissues, and are the main molecular target of the endocannabinoid ligand (binding molecule), Anandamide, as well as its mimetic phytocannabinoid, THC. One other main endocannabinoid is 2-Arachidonoylglycerol (2-AG) which is active at both cannabinoid receptors, along with its own mimetic phytocannabinoid, CBD. 2-AG and CBD are involved in the regulation of appetite, immune system functions and pain management.[10][11][12]

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Basic overview[edit]

The endocannabinoid system, broadly speaking, includes:
The endogenous arachidonate-based lipids, anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG); these are known as "endocannabinoids" and are physiological ligands for the cannabinoid receptors. Endocannabinoids are all eicosanoids.[13]
The enzymes that synthesize and degrade the endocannabinoids, such as fatty acid amide hydrolase or monoacylglycerol lipase.
The cannabinoid receptors CB1 and CB2, two G protein-coupled receptors that are located in the central and peripheral nervous systems.

The neurons, neural pathways, and other cells where these molecules, enzymes, and one or both cannabinoid receptor types are all colocalized from the endocannabinoid system.

The endocannabinoid system has been studied using genetic and pharmacological methods. These studies have revealed that cannabinoids act as neuromodulators[14][15][16] for a variety of processes, including motor learning,[17] appetite,[18] and pain sensation,[19] among other cognitive and physical processes. The localization of the CB1 receptor in the endocannabinoid system has a very large degree of overlap with the orexinergic projection system, which mediates many of the same functions, both physical and cognitive.[20] Moreover, CB1 is colocalized on orexin projection neurons in the lateral hypothalamus and many output structures of the orexin system,[20][21] where the CB1 and orexin receptor 1 (OX1) receptors physically and functionally join together to form the CB1–OX1 receptor heterodimer.[20][22][23]

Expression of receptors[edit]

For more details on receptor localization, see Cannabinoid receptor type 1 (CB1) and Cannabinoid receptor type 2 (CB2).

Cannabinoid binding sites exist throughout the central and peripheral nervous systems. The two most relevant receptors for cannabinoids are the CB1 and CB2 receptors, which are expressed predominantly in the brain and immune system respectively.[24] Density of expression varies based on species and correlates with the efficacy that cannabinoids will have in modulating specific aspects of behavior related to the site of expression. For

example, in rodents, the highest concentration of cannabinoid binding sites are in the basal ganglia and cerebellum, regions of the brain involved in the initiation and coordination of movement.[25] In humans, cannabinoid receptors exist in much lower concentration in these regions, which helps explain why cannabinoids possess a greater efficacy in altering rodent motor movements than they do in humans.

A recent analysis of cannabinoid binding in CB1 and CB2 receptor knockout mice found cannabinoid responsiveness even when these receptors were not being expressed, indicating that an additional binding receptor may be present in the brain.[25] Binding has been demonstrated by 2-arachidonoylglycerol (2-AG) on the TRPV1 receptor suggesting that this receptor may be a candidate for the established response.[26]

In addition to CB1 and CB2, certain orphan receptors are known to bind endocannabinoids as well, including GPR18, GPR55 (a regulator of neuroimmune function), and GPR119. CB1 has also been noted to form a functional human receptor heterodimer in orexin neurons with OX1, the CB1–OX1 receptor, which mediates feeding behavior and certain physical processes such as cannabinoid-induced pressor responses which are known to occur through signaling in the rostral ventrolateral medulla.[27][28]

Endocannabinoid synthesis, release, and degradation[edit]

During neurotransmission, the pre-synaptic neuron releases neurotransmitters into the synaptic cleft which bind to cognate receptors expressed on the post-synaptic neuron. Based upon the interaction between the transmitter and receptor, neurotransmitters may trigger a variety of effects in the post-synaptic cell, such as excitation, inhibition, or the initiation of second messenger cascades. Based on the cell, these effects may result in the on-site synthesis of endogenous cannabinoids anandamide or 2-AG by a process that is not entirely clear, but results from an elevation in intracellular calcium.[24] Expression appears to be exclusive, so that both types of endocannabinoids are not co-synthesized. This exclusion is based on synthesis-specific channel activation: a recent study found that in the bed nucleus of the stria terminalis, calcium entry through voltage-sensitive calcium channels produced an L-type current resulting in 2-AG production, while activation of mGluR1/5 receptors triggered the synthesis of anandamide.[26]

Evidence suggests that the depolarization-induced influx of calcium into the post-synaptic neuron causes the activation of an enzyme called transacylase. This enzyme is suggested to catalyze the first step of endocannabinoid biosynthesis by converting phosphatidylethanolamine, a membrane-resident phospholipid, into N-acyl-phosphatidylethanolamine (NAPE). Experiments have shown that phospholipase D cleaves NAPE to yield anandamide.[29][30] This process is mediated by bile acids.[31] In NAPE-phospholipase D (NAPEPLD)-knockout mice, cleavage of NAPE is reduced in low calcium concentrations, but not abolished, suggesting multiple, distinct pathways are involved in anandamide synthesis.[32] The synthesis of 2-AG is less established and warrants further research.

Once released into the extracellular space by a putative endocannabinoid transporter, messengers are vulnerable to glial cell inactivation. Endocannabinoids are taken up by a transporter on the glial cell and degraded by fatty acid amide hydrolase (FAAH), which cleaves anandamide into arachidonic acid and ethanolamine or monoacylglycerol lipase (MAGL), and 2-AG into arachidonic acid and glycerol.[33] While arachidonic acid is a substrate for leukotriene and prostaglandin synthesis, it is unclear whether this degradative byproduct has unique functions in the central nervous system.[34][35] Emerging data in the field also points to FAAH being expressed in postsynaptic neurons complementary to presynaptic neurons expressing cannabinoid receptors, supporting the conclusion that it is major contributor to the clearance and inactivation of anandamide and 2-AG after endocannabinoid reuptake.[25] A neuropharmacological study demonstrated that an inhibitor of FAAH (URB597) selectively increases anandamide levels in the brain of rodents and primates. Such approaches could lead to the development of new drugs with analgesic, anxiolytic-like and antidepressant-like effects, which are not accompanied by overt signs of abuse liability.[36]

Binding and intracellular effects[edit]

Cannabinoid receptors are G-protein coupled receptors located on the pre-synaptic membrane. While there have been some papers that have linked concurrent stimulation of dopamine and CB1 receptors to an acute rise in cyclic adenosine monophosphate (cAMP) production, it is generally accepted that CB1 activation via cannabinoids causes a decrease in cAMP concentration by inhibition of adenylyl cyclase and a rise in the concentration of mitogen-activated protein kinase (MAP kinase).[13][25] The relative potency of different cannabinoids in inhibition of adenylyl cyclase correlates with their varying efficacy in behavioral assays. This inhibition of cAMP is followed by phosphorylation and subsequent activation of not only a suite of MAP kinases (p38/p42/p44), but also the PI3/PKB and MEK/ERK pathway (Galve-Roperh et al., 2002; Davis et al., 2005; Jones et al., 2005; Graham et al., 2006). Results from rat hippocampal gene chip data after acute administration of tetrahydrocannabinol (THC) showed an increase in the expression of transcripts encoding myelin basic protein, endoplasmic proteins, cytochrome oxidase, and two cell adhesion molecules: NCAM, and SC1; decreases in expression were seen in both calmodulin and ribosomal RNAs (Kittler et al., 2000). In addition, CB1 activation has been demonstrated to increase the activity of transcription factors like c-Fos and Krox-24 (Graham et al., 2006).

Binding and neuronal excitability[edit]

This section provides insufficient context for those unfamiliar with the subject. Please help improve the article with a good introductory style. (January 2014) (Learn how and when to remove this template message)

The molecular mechanisms of CB1-mediated changes to the membrane voltage have also been studied in detail. Cannabinoids reduce calcium influx by blocking the activity of voltage-dependent N-, P/Q- and L-type calcium channels.[37][38] In addition to acting on calcium channels, activation of Gi/o and Gs, the two most commonly coupled G-proteins to cannabinoid receptors, has been shown to modulate potassium channel activity. Recent studies have found that CB1 activation specifically facilitates potassium ion flux through GIRKs, a family of potassium channels.[38] Immunohistochemistry experiments demonstrated that CB1 is co-localized with GIRK and Kv1.4 potassium channels, suggesting that these two may interact in physiological contexts.[39]

In the central nervous system, CB1 receptors influence neuronal excitability, reducing the incoming synaptic input.[40] This mechanism, known as presynaptic inhibition, occurs when a postsynaptic neuron releases endocannabinoids in retrograde transmission, which then bind to cannabinoid receptors on the presynaptic terminal. CB1 receptors then reduce the amount of neurotransmitter released, so that subsequent excitation in the presynaptic neuron results in diminished effects on the postsynaptic neuron. It is likely that presynaptic inhibition uses many of the same ion channel mechanisms listed above, although recent evidence has shown that CB1 receptors can also regulate neurotransmitter release by a non-ion channel mechanism, i.e. through Gi/o-mediated inhibition of adenylyl cyclase and protein kinase A.[41] Direct effects of CB1 receptors on membrane excitability have been reported, and strongly impact the firing of cortical neurons.[42] A series of behavioral experiments demonstrated that NMDAR, an ionotropic glutamate receptor, and the metabotropic glutamate receptors (mGluRs) work in concert with CB1 to induce analgesia in mice, although the mechanism underlying this effect is unclear.[citation needed]

Functions of the endocannabinoid system[edit]

Memory[edit]

Mice treated with tetrahydrocannabinol (THC) show suppression of long-term potentiation in the hippocampus, a process that is essential for the formation and storage of long-term memory.[43] These results concur with anecdotal evidence suggesting that smoking Cannabis impairs short-term memory.[44] Consistent with this finding, mice without the CB1 receptor show enhanced memory and long-term potentiation indicating that the endocannabinoid system may play a pivotal role in the extinction of old memories. One study found that the high-dose treatment of rats with the synthetic cannabinoid HU-210 over several weeks resulted in stimulation of neural growth in the rats' hippocampus region, a part of the limbic system playing a part in the formation of declarative and spatial memories, but did not investigate the effects on short-term or long-term memory.[45] Taken together, these findings suggest that the effects of endocannabinoids on the various brain networks involved in learning and memory may vary.

Role in hippocampal neurogenesis[edit]

In the adult brain, the endocannabinoid system facilitates the neurogenesis of hippocampal granule cells.[45][46] In the subgranular zone of the dentate gyrus, multipotent neural progenitors (NP) give rise to daughter cells that, over the course of several weeks, mature into granule cells whose axons project to and synapse onto dendrites on the CA3 region.[47] NPs in the hippocampus have been shown to possess fatty acid amide hydrolase (FAAH) and express CB1 and utilize 2-AG.[46] Intriguingly, CB1 activation by endogenous or exogenous cannabinoids promote NP proliferation and differentiation; this activation is absent in CB1 knockouts and abolished in the presence of antagonist.[45][46]

Induction of synaptic depression[edit]

The inhibitory effects of cannabinoid receptor stimulation on neurotransmitter release have caused this system to be connected to various forms of depressant plasticity. A recent study conducted with the bed nucleus of the stria terminalis found that the endurance of the depressant effects was mediated by two different signaling pathways based on the type of receptor activated. 2-AG was found to act on presynaptic CB1 receptors to mediate retrograde short-term depression (STD) following activation of L-type calcium currents, while anandamide was synthesized after mGluR5 activation and triggered autocrine signalling onto postsynapic TRPV1 receptors that induced long-term depression (LTD). Similar post-synaptic receptor dependencies were found in the striatum, but here both effects relied on presynaptic CB1 receptors.[26] These findings provide the brain a direct mechanism to selectively inhibit neuronal excitability over variable time scales. By selectively internalizing different receptors, the brain may limit the production of specific endocannabinoids to favor a time scale in accordance with its needs.

Appetite[edit]

Evidence for the role of the endocannabinoid system in food-seeking behavior comes from a variety of cannabinoid studies. Emerging data suggests that THC acts via CB1 receptors in the hypothalamic nuclei to directly increase appetite.[48] It is thought that hypothalamic neurons tonically produce endocannabinoids that work to tightly regulate hunger. The amount of endocannabinoids produced is inversely correlated with the amount of leptin in the blood.[49] For example, mice without leptin not only become massively obese but express abnormally high levels of hypothalamic endocannabinoids as a compensatory mechanism.[18] Similarly, when these mice were treated with an endocannabinoid inverse agonists, such as rimonabant, food intake was reduced.[18] When the CB1 receptor is knocked out in mice, these animals tend to be leaner and less hungry than wild-type mice. A related study examined the effect of THC on the hedonic (pleasure) value of food and found enhanced dopamine release in the nucleus accumbens and increased pleasure-related behavior after administration of a sucrose solution.[50] A related study found that endocannabinoids affect taste perception in taste cells[51] In taste cells, endocannabinoids were shown to selectively enhance the strength of neural signaling for sweet tastes, whereas leptin decreased the strength of this same response. While there is need for more research, these results suggest that cannabinoid activity in the hypothalamus and nucleus accumbens is related to appetitive, food-seeking behavior.[48]

Energy balance and metabolism[edit]

The endocannabinoid system has been shown to have a homeostatic role by controlling several metabolic functions, such as energy storage and nutrient transport. It acts on peripheral tissues such as adipocytes, hepatocytes, the gastrointestinal tract, the skeletal muscles and the endocrine pancreas. It has also been implied in modulating insulin sensitivity. Through all of this, the endocannabinoid system may play a role in clinical conditions, such as obesity, diabetes, and atherosclerosis, which may also give it a cardiovascular role.[52]

Stress response[edit]

While the secretion of glucocorticoids in response to stressful stimuli is an adaptive response necessary for an organism to respond appropriately to a stressor, persistent secretion may be harmful. The endocannabinoid system has been implicated in the habituation of the hypothalamic-pituitary-adrenal axis (HPA axis) to repeated exposure to restraint stress. Studies have demonstrated differential synthesis of anandamide and 2-AG during tonic stress. A decrease of anandamide was found along the axis that contributed to basal hypersecretion of corticosterone; in contrast, an increase of 2-AG was found in the amygdala after repeated stress, which was negatively correlated to magnitude of the corticosterone response. All effects were abolished by the CB1 antagonist AM251, supporting the conclusion that these effects were cannabinoid-receptor dependent.[53] These findings show that anandamide and 2-AG divergently regulate the HPA axis response to stress: while habituation of the stress-induced HPA axis via 2-AG prevents excessive secretion of glucocorticoids to non-threatening stimuli, the increase of basal corticosterone secretion resulting from decreased anandamide allows for a facilitated response of the HPA axis to novel stimuli.

Exploration, social behavior, and anxiety[edit]

.[54] These contrasting effects reveal the importance of the endocannabinoid system in regulating anxiety-dependent behavior. Results suggest that glutamatergic cannabinoid receptors are not only responsible for mediating aggression, but produce an anxiolytic-like function by inhibiting excessive arousal: excessive excitation produces anxiety that limited the mice from exploring both animate and inanimate objects. In contrast, GABAergic neurons appear to control an anxiogenic-like function by limiting inhibitory transmitter release. Taken together, these two sets of neurons appear to help regulate the organism's overall sense of arousal during novel situations.

Immune function[edit]

Evidence suggests that endocannabinoids may function as both neuromodulators and immunomodulators in the immune system. Here, they seem to serve an autoprotective role to ameliorate muscle spasms, inflammation, and other symptoms of multiple sclerosis and skeletal muscle spasms.[13] Functionally, the activation of cannabinoid receptors has been demonstrated to play a role in the activation of GTPases in macrophages, neutrophils, and BM cells. These receptors have also been implicated in the proper migration of B cells into the marginal zone (MZ) and the regulation of healthy IgM levels.[55] Interestingly, some disorders seem to trigger an upregulation of cannabinoid receptors selectively in cells or tissues related to symptom relief and inhibition of disease progression, such as in that rodent neuropathic pain model, where receptors are increased in the spinal cord

microglia, dorsal root ganglion, and thalamic neurons.[24]

Multiple sclerosis[edit]

Historical records from ancient China and Greece suggest that preparations of Cannabis indica were commonly prescribed to ameliorate multiple sclerosis-like symptoms such as tremors and muscle pain. Modern research has confirmed these effects in a study on diseased mice, wherein both endogenous and exogenous agonists showed ameliorating effects on tremor and spasticity. It remains to be seen whether pharmaceutical preparations such as dronabinol have the same effects in humans.[56][57] Due to increasing use of medical Cannabis and rising incidence of multiple sclerosis patients who self-medicate with the drug, there has been much interest in exploiting the endocannabinoid system in the cerebellum to provide a legal and effective relief.[44] In mouse models of multiple sclerosis, there is a profound reduction and reorganization of CB1 receptors in the cerebellum.[58] Serial sections of cerebellar tissue subjected to immunohistochemistry revealed that this aberrant expression occurred during the relapse phase but returned to normal during the remitting phase of the disease.[58] Other studies suggest that CB1 agonists promote the survival of oligodendrocytes in vitro in the absence of growth and trophic factors; in addition, these agonist have been shown to promote mRNA expression of myelin lipid protein. (Kittler et al., 2000; Mollna-Holgado et al., 2002). Taken together, these studies point to the exciting possibility that cannabinoid treatment may not only be able to attenuate the symptoms of multiple sclerosis but also improve oligodendrocyte function (reviewed in Pertwee, 2001; Mollna-Holgado et al., 2002). 2-AG stimulates proliferation of a microglial cell line by a CB2 receptor dependent mechanism, and the number of microglial cells is increased in multiple sclerosis.[59]

Female reproduction[edit]

See also: Cannabis in pregnancy

The developing embryo expresses cannabinoid receptors early in development that are responsive to anandamide secreted in the uterus. This signaling is important in regulating the timing of embryonic implantation and uterine receptivity. In mice, it has been shown that anandamide modulates the probability of implantation to the uterine wall. For example, in humans, the likelihood of miscarriage increases if uterine anandamide levels are too high or low.[60] These results suggest that intake of exogenous cannabinoids (e.g. marijuana) can decrease the likelihood for pregnancy for women with high anandamide levels, and alternatively, it can increase the likelihood for pregnancy in women whose anandamide levels were too low.[61][62]

Autonomic nervous system[edit]

Peripheral expression of cannabinoid receptors led researchers to investigate the role of cannabinoids in the autonomic nervous system. Research found that the CB1 receptor is expressed presynaptically by motor neurons that innervate visceral organs. Cannabinoid-mediated inhibition of electric potentials results in a reduction in noradrenaline release from sympathetic nervous system nerves. Other studies have found similar effects in endocannabinoid regulation of intestinal motility, including the innervation of smooth muscles associated with the digestive, urinary, and reproductive systems.[25]

Analgesia[edit]

At the spinal cord, cannabinoids suppress noxious-stimulus-evoked responses of neurons in the dorsal horn, possibly by modulating descending noradrenaline input from the brainstem.[25] As many of these fibers are primarily GABAergic, cannabinoid stimulation in the spinal column results in disinhibition that should increase noradrenaline release and attenuation of noxious-stimuli-processing in the periphery and dorsal root ganglion.

The endocannabinoid most researched in pain is palmitoylethanolamide. Palmitoylethanolamide is a fatty amine related to anandamide, but saturated and although initially it was thought that palmitoylethanolamide would bind to the CB1 and the CB2 receptor, later it was found that the most important receptors are the PPAR-alpha receptor, the TRPV receptor and the GPR55 receptor. Palmitoylethanolamide has been evaluated for its analgesic actions in a great variety of pain indications[63] and found to be safe and effective. Basically these data are proof of concept for endocannabinoids and related fatty amines to be therapeutically useful analgesics; palmitoylethanolamide is available under the brand names Normast and PeaPure as nutraceuticals.

Endocannabinoids are involved in placebo induced analgesia responses.[64]

Thermoregulation[edit]

Anandamide and N-arachidonoyl dopamine (NADA) have been shown to act on temperature-sensing TRPV1 channels, which are involved in thermoregulation.[65] TRPV1 is activated by the exogenous ligand capsaicin, the active component of chili peppers, which is structurally similar to endocannabinoids. NADA activates the TRPV1 channel with an EC50 of approximately of 50 nM.[clarify] The high potency makes it the putative endogenous TRPV1 agonist.[66] Anandamide has also been found to activate TRPV1 on sensory neuron terminals, and subsequently cause vasodilation.[25] TRPV1 may also be activated by methanandamide and arachidonyl-2'-chloroethylamide (ACEA).[13]

Sleep[edit]

Increased endocannabinoid signaling within the central nervous system promotes sleep-inducing effects. Intercerebroventricular administration of anandamide in rats has been shown to decrease wakefulness and increase slow-wave sleep and REM sleep.[67] Administration of anandamide into the basal forebrain of rats has also been shown to increase levels of adenosine, which plays a role in promoting sleep and suppressing arousal.[68] REM sleep deprivation in rats has been demonstrated to increase CB1 receptor expression in the central nervous system.[69] Furthermore, anandamide levels possess a circadian rhythm in the rat, with levels being higher in the light phase of the day, which is when rats are usually asleep or less active, since they are nocturnal.[70]

Physical exercise[edit]

Anandamide is an endogenous cannabinoid neurotransmitter that binds to cannabinoid receptors.[6] It has been shown that aerobic exercise causes an increase in plasma anandamide levels, where the magnitude of this increase is highest at moderate exercise intensity (i.e., exercising at ~70–80% maximum heart rate).[6] Increases in plasma anandamide levels are associated with psychoactive effects because anandamide is able to cross the blood–brain barrier and act within the central nervous system.[6] Thus, because anandamide is a euphoriant and aerobic exercise is associated with euphoric effects, it has been proposed that anandamide partly mediates the short-term mood-lifting effects of exercise (e.g., the euphoria of a runner's high) via exercise-induced increases in its synthesis.[7][6]

In mice it was demonstrated that certain features of a runner's high depend on cannabinoid receptors. Pharmacological or genetic disruption of

cannabinoid signaling via cannabinoid receptors prevents the analgesic and anxiety-reducing effects of running.[71][non-primary source needed]

Experimental use of CB1 -/- phenotype[edit]

Neuroscientists often utilize transgenic CB1 knockout mice to discern novel roles for the endocannabinoid system. While CB1 knockout mice are healthy and live into adulthood, there are significant differences between CB1 knockout and wild-type mice. When subjected to a high-fat diet, CB1 knockout mice tend to be about sixty percent leaner and slightly less hungry than wildtype.[72] Compared to wildtype, CB1 knockout mice exhibit severe deficits in motor learning, memory retrieval, and increased difficulty in completing the Morris water maze.[17][73][74] There is also evidence indicating that these knockout animals have an increased incidence and severity of stroke and seizure.[75][76]

Endocannabinoids in plants[edit]

The endocannabinoid system is by molecular phylogenetic distribution of apparently ancient lipids in the plant kingdom, indicative of biosynthetic plasticity and potential physiological roles of endocannabinoid-like lipids in plants,[77] and detection of arachidonic acid (AA) indicates chemotaxonomic connections between monophyletic groups with common ancestor dates to around 420 million years ago (silurian; devonian). The phylogenetic distribution of these lipids may be a consequence of interactions/adaptations to the surrounding conditions such as chemical plant-pollinator interactions, communication and defense mechanisms. The two novel EC-like molecules derived from the eicosatetraenoic acid juniperonic acid, an omega-3 structural isomer of AA, namely juniperoyl ethanolamide and 2-juniperoyl glycerol (1/2-AG) in gymnosperms, lycophytes and few monilophytes, show AA is an evolutionarily conserved signalling molecule that acts in plants in response to stress similar to that in animal systems.[78]

See also[edit]
Endocannabinoid enhancer
Endocannabinoid reuptake inhibitor
Cannabinoid receptor antagonist
Jasmonate

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External links[edit]

Homepage of the ICRS - The International Cannabinoid Research Society
Homepage of the ECSN – The Endocannabinoid System Network

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Cannabinoid receptor modulators
https://en.wikipedia.org/wiki/Endocannabinoid_system

Did you know, it is estimated that hemp has approximately 25,000 uses? From food, paint and fuel to clothing and construction materials, hemp is used. There are even hemp fibres in your Red Rose® and Lipton® tea bags. And several cars made today contain hemp.

The oldest relic of human industry is a piece of hemp fabric (canvas) found in ancient Mesopotamia dating back to approximately 8000 B.C. The oldest surviving piece of paper was made over 2000 years ago in China and was also made from hemp fibre. In 2500 B.C. the pharaohs used hemp in the construction of the great pyramids.

Hemp was so important in England in the 16th century that King Henry VIII passed a law in 1553 which fined farmers who failed to grow at least one quarter acre of hemp for every 60 acres of arable land they owned. There was even a time in history for over 200 years when you could pay your taxes in America with hemp. In 1850 there were more than 8,300 hemp farms in the United States.

Every 3.6 seconds someone in the world dies of hunger. Hemp seeds are the most nutritious and economical solution to end world hunger. With an 80 percent concentration of "good fats" our bodies need for good health maintenance and protein with all eight amino acids plus optimum dietary fibre, hemp truly is a "perfect balance" food source.

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Nature has provided the essential nutrients for restoring and maintaining optimal health in a power-packed seed that is gluten, cholesterol and sugar free. Pure, natural, raw shelled hemp seed - the best single food source of protein, essential fats, vitamins and enzymes on the planet.

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in a power-packed seed that is gluten, cholesterol and sugar free. Pure, natural,
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THE REAL REASON CANNABIS HAS BEEN OUTLAWED HAS NOTHING
TO DO WITH ITS EFFECTS ON THE MIND AND BODY.

Doug Yurchey

MARIJUANA is DANGEROUS. Pot is NOT harmful to the human body or mind. Marijuana does NOT pose a threat to the general public. Marijuana is very much a danger to the oil companies, alcohol, tobacco industries and a large number of chemical corporations. Various big businesses, with plenty of dollars and influence, have suppressed the truth from the people.

The truth is if marijuana was utilized for its vast array of commercial products, it would create an industrial atomic bomb! Entrepreneurs have not been educated on the product potential of pot. The super rich have conspired to spread misinformation about an extremely versatile plant that, if used properly, would ruin their companies.

Where did the word 'marijuana' come from? In the mid 1930s, the M-word was created to tarnish the good image and phenomenal history of the hemp plant...as you will read. The facts cited here, with references, are generally verifiable in the Encyclopedia Britannica which was printed on hemp paper for 150 years:

* All schoolbooks were made from hemp or flax paper until the 1880s; Hemp Paper Reconsidered, Jack Frazier, 1974.

* It was LEGAL TO PAY TAXES WITH HEMP in America from 1631 until the early 1800s; LA Times, Aug. 12, 1981.

* REFUSING TO GROW HEMP in America during the 17th and 18th Centuries WAS AGAINST THE LAW! You could be jailed in Virginia for refusing to grow hemp from 1763 to 1769; Hemp in Colonial Virginia, G. M. Herdon.

"I grew Hemp", George Washington

* George Washington, Thomas Jefferson and other founding fathers GREW HEMP; Washington and Jefferson Diaries. Jefferson smuggled hemp seeds from China to France then to America.

* Benjamin Franklin owned one of the first paper mills in America and it processed hemp. Also, the War of 1812 was fought over hemp. Napoleon wanted to cut off Moscow's export to England; Emperor Wears No Clothes, Jack Herer.

* For thousands of years, 90% of all ships' sails and rope were made from hemp. The word 'canvas' is Dutch for cannabis; Webster's New World Dictionary.

* 80% of all textiles, fabrics, clothes, linen, drapes, bed sheets, etc. were made from hemp until the 1820s with the introduction of the cotton gin.

* The first Bibles, maps, charts, Betsy Ross's flag, the first drafts of the Declaration of Independence and the Constitution were made from hemp; U.S. Government Archives.

* The first crop grown in many states was hemp. 1850 was a peak year for Kentucky producing 40,000 tons. Hemp was the largest cash crop until the 20th Century; State Archives.

* Oldest known records of hemp farming go back 5000 years in China, although hemp industrialization probably goes back to ancient Egypt.

* Rembrants, Gainsboroughs, Van Goghs as well as most early canvas paintings were principally painted on hemp linen.

* In 1916, the U.S. Government predicted that by the 1940s all paper would come from hemp and that no more trees need to be cut down. Government studies report that 1 acre of hemp equals 4.1 acres of trees. Plans were in the works to implement such programs; Department of Agriculture

* Quality paints and varnishes were made from hemp seed oil until 1937. 58,000 tons of hemp seeds were used in America for paint products in 1935; Sherman Williams Paint Co. testimony before Congress against the 1937 Marijuana Tax Act.

* Henry Ford's first Model-T was built to run on hemp gasoline and the CAR ITSELF WAS CONSTRUCTED FROM HEMP! On his large estate, Ford was photographed among his hemp fields. The car, 'grown from the soil,' had hemp plastic panels whose impact strength was 10 times stronger than steel; Popular Mechanics, 1941.

* Hemp called 'Billion Dollar Crop.' It was the first time a cash crop had a business potential to exceed a billion dollars; Popular Mechanics, Feb., 1938.

* Mechanical Engineering Magazine (Feb. 1938) published an article entitled 'The Most Profitable and Desirable Crop that Can be Grown.' It stated that if hemp was cultivated using 20th Century technology, it would be the single largest agricultural crop in the U.S. and the rest of the world.

The following information comes directly from the United States Department of Agriculture's 1942 14-minute film encouraging and instructing 'patriotic American farmers' to grow 350,000 acres of hemp each year for the war effort:

'...(When) Grecian temples were new, hemp was already old in the service of mankind. For thousands of years, even then, this plant had been grown for cordage and cloth in China and elsewhere in the East. For centuries prior to about 1850, all the ships that sailed the western seas were rigged with hempen rope and sails. For the sailor, no less than the hangman, hemp was indispensable...

...Now with Philippine and East Indian sources of hemp in the hands of the Japanese...American hemp must meet the needs of our Army and Navy as well as of our industries...

...the Navy's rapidly dwindling reserves. When that is gone, American hemp will go on duty again; hemp for mooring ships; hemp for tow lines; hemp for tackle and gear; hemp for countless naval uses both on ship and shore. Just as in the days when Old Ironsides sailed the seas victorious with her hempen shrouds and hempen sails. Hemp for victory!'

Certified proof from the Library of Congress; found by the research of Jack Herer, refuting claims of other government agencies that the 1942 USDA film 'Hemp for Victory' did not exist.

Hemp cultivation and production do not harm the environment. The USDA Bulletin #404 concluded that Hemp produces 4 times as much pulp with at least 4 to 7 times less pollution. From Popular Mechanics, Feb. 1938:

'It has a short growing season...It can be grown in any state...The long roots penetrate and break the soil to leave it in perfect condition for the next year's crop. The dense shock of leaves, 8 to 12 feet above the ground, chokes out weeds.
...hemp, this new crop can add immeasurably to American agriculture and industry.'

In the 1930s, innovations in farm machinery would have caused an industrial revolution when applied to hemp. This single resource could have created millions of new jobs generating thousands of quality products. Hemp, if not made illegal, would have brought America out of the Great Depression.

William Randolph Hearst (Citizen Kane) and the Hearst Paper Manufacturing Division of Kimberly Clark owned vast acreage of timberlands. The Hearst Company supplied most paper products. Patty Hearst's grandfather, a destroyer of nature for his own personal profit, stood to lose billions because of hemp.

In 1937, Dupont patented the processes to make plastics from oil and coal. Dupont's Annual Report urged stockholders to invest in its new petrochemical division. Synthetics such as plastics, cellophane, celluloid, methanol, nylon, rayon, Dacron, etc., could now be made from oil. Natural hemp industrialization would have ruined over 80% of Dupont's business.

THE CONSPIRACY

Andrew Mellon became Hoover's Secretary of the Treasury and Dupont's primary investor. He appointed his future nephew-in-law, Harry J. Anslinger, to head the Federal Bureau of Narcotics and Dangerous Drugs.

Secret meetings were held by these financial tycoons. Hemp was declared dangerous and a threat to their billion dollar enterprises. For their dynasties to remain intact, hemp had to go. These men took an obscure Mexican slang word: 'marihuana' and pushed it into the consciousness of America.

MEDIA MANIPULATION

A media blitz of 'yellow journalism' raged in the late 1920s and 1930s. Hearst's newspapers ran stories emphasizing the horrors of marihuana. The menace of marihuana made headlines. Readers learned that it was responsible for everything from car accidents to loose morality.

Films like 'Reefer Madness' (1936), 'Marihuana: Assassin of Youth' (1935) and 'Marihuana: The Devil's Weed' (1936) were propaganda designed by these industrialists to create an enemy. Their purpose was to gain public support so that anti-marihuana laws could be passed.

Examine the following quotes from 'The Burning Question' aka REEFER MADNESS:

• a violent narcotic.

• acts of shocking violence.

• incurable insanity.

• soul-destroying effects.

• under the influence of the drug he killed his entire family with an ax.

• more vicious, more deadly even than these soul-destroying drugs (heroin, cocaine) is the menace of marihuana!

Reefer Madness did not end with the usual 'the end.' The film concluded with these words plastered on the screen: TELL YOUR CHILDREN.

In the 1930s, people were very naive; even to the point of ignorance. The masses were like sheep waiting to be led by the few in power. They did not challenge authority. If the news was in print or on the radio, they believed it had to be true. They told their children and their children grew up to be the parents of the baby-boomers.

On April 14, 1937, the Prohibitive Marihuana Tax Law or the bill that outlawed hemp was directly brought to the House Ways and Means Committee. This committee is the only one that can introduce a bill to the House floor without it being debated by other committees. The Chairman of the Ways and Means, Robert Doughton, was a Dupont supporter. He insured that the bill would pass Congress.

Dr. James Woodward, a physician and attorney, testified too late on behalf of the American Medical Association. He told the committee that the reason the AMA had not denounced the Marihuana Tax Law sooner was that the Association had just discovered that marihuana was hemp.

Few people, at the time, realized that the deadly menace they had been reading about on Hearst's front pages was in fact passive hemp. The AMA understood cannabis to be a MEDICINE found in numerous healing products sold over the last hundred years.

In September of 1937, hemp became illegal. The most useful crop known became a drug and our planet has been suffering ever since.

Congress banned hemp because it was said to be the most violence-causing drug known. Anslinger, head of the Drug Commission for 31 years, promoted the idea that marihuana made users act extremely violent. In the 1950s, under the Communist threat of McCarthyism, Anslinger now said the exact opposite. Marijuana will pacify you so much that soldiers would not want to fight.

Today, our planet is in desperate trouble. Earth is suffocating as large tracts of rain forests disappear. Pollution, poisons and chemicals are killing people. These great problems could be reversed if we industrialized hemp. Natural biomass could provide all of the planet's energy needs that are currently supplied by fossil fuels. We have consumed 80% of our oil and gas reserves. We need a renewable resource. Hemp could be the solution to soaring gas prices.

Hemp

THE WONDER PLANT

Hemp has a higher quality fiber than wood fiber. Far fewer caustic chemicals are required to make paper from hemp than from trees. Hemp paper does not turn yellow and is very durable. The plant grows quickly to maturity in a season where trees take a lifetime.

ALL PLASTIC PRODUCTS SHOULD BE MADE FROM HEMP SEED OIL. Hempen plastics are biodegradable! Over time, they would break down and not harm the environment. Oil-based plastics, the ones we are very familiar with, help ruin nature; they do not break down and will do great harm in the future. The process to produce the vast array of natural (hempen) plastics will not ruin the rivers as Dupont and other petrochemical companies have done. Ecology does not fit in with the plans of the Oil Industry and the political machine. Hemp products are safe and natural.

MEDICINES SHOULD BE MADE FROM HEMP. We should go back to the days when the AMA supported cannabis cures. 'Medical Marijuana' is given out legally to only a handful of people while the rest of us are forced into a system that relies on chemicals. Pot is only healthy for the human body.

WORLD HUNGER COULD END. A large variety of food products can be generated from hemp. The seeds contain one of the highest sources of protein in nature. ALSO: They have two essential fatty acids that clean your body of cholesterol. These essential fatty acids are not found anywhere else in nature! Consuming pot seeds is the best thing you could do for your body. Eat uncooked hemp seeds.

CLOTHES SHOULD BE MADE FROM HEMP. Hemp clothing is extremely strong and durable over time. You could hand clothing, made from pot, down to your grandchildren. Today, there are American companies that make hemp clothing; usually 50% hemp. Hemp fabrics should be everywhere. Instead, they are almost underground. Superior hemp products are not allowed to advertise on fascist television. Kentucky, once the top hemp producing state, made it ILLEGAL TO WEAR hemp clothing! Can you imagine being thrown into jail for wearing quality jeans?

The world is crazy...but that does not mean you have to join the insanity. Get together. Spread the news. Tell people, and that includes your children, the truth. Use hemp products. Eliminate the word 'marijuana.' Realize the history that created it. Make it politically incorrect to say or print the M-word. Fight against the propaganda (designed to favor the agenda of the super rich) and the bullshit. Hemp must be utilized in the future. We need a clean energy source to save our planet. INDUSTRIALIZE HEMP!

The liquor, tobacco and oil companies fund more than a million dollars a day to Partnership for a Drug-Free America and other similar agencies. We have all seen their commercials. Now, their motto is: 'It's more dangerous than we thought.' Lies from the powerful corporations, that began with Hearst, are still alive and well today.

The brainwashing continues. Now, the commercials say: If you buy a joint, you contribute to murders and gang wars. The latest anti-pot commercials say: If you buy a joint...you are promoting TERRORISM! The new enemy (terrorism) has paved the road to brainwash you any way THEY see fit.

There is only one enemy; the friendly people you pay your taxes to; the war-makers and nature destroyers. With your funding, they are killing the world right in front of your eyes. HALF A MILLION DEATHS EACH YEAR ARE CAUSED BY TOBACCO. HALF A MILLION DEATHS EACH YEAR ARE CAUSED BY ALCOHOL. NO ONE HAS EVER, EVER DIED FROM SMOKING POT!! In the entire history of the human race, not one death can be attributed to cannabis. Our society has outlawed grass but condones the use of the KILLERS: TOBACCO and ALCOHOL. Hemp should be declassified and placed in DRUG stores to relieve stress. Hardening and constriction of the arteries are bad; but hemp usage actually enlarges the arteries...which is a healthy condition. We have been so conditioned to think that: Smoking is harmful. That is NOT the case for passive pot.

Ingesting THC, hemp's active agent, has a positive effect; relieving asthma and glaucoma. A joint tends to alleviate the nausea caused by chemotherapy. You are able to eat on hemp. This is a healthy state of being.

(One personal note: During the pregnancy of my wife, she was having some difficulty gaining weight. We were in the hospital. A nurse called us to one side and said: 'Off the record, if you smoke pot...you'd get something called the munchies and you'll gain weight.' I swear that is a true story). [Using Medifast coupons to counteract the results of the weight gain later on is a different story.]

The stereotype for a pothead is similar to a drunk, bubble-brain. Yet, the truth is one's creative abilities can be enhanced under its influence. The perception of time slightly slows and one can become more sensitive. You can more appreciate all arts; be closer to nature and generally FEEL more under the influence of cannabis. It is, in fact, the exact opposite state of mind and body as the drunken state. You can be more aware with pot.

The pot plant is an ALIEN plant. There is physical evidence that cannabis is not like any other plant on this planet. One could conclude that it was brought here for the benefit of humanity. Hemp is the ONLY plant where the males appear one way and the females appear very different, physically! No one ever speaks of males and females in regard to the plant kingdom because plants do not show their sexes; except for cannabis. To determine what sex a certain, normal, Earthly plant is: You have to look internally, at its DNA. A male blade of grass (physically) looks exactly like a female blade of grass. The hemp plant has an intense sexuallity. Growers know to kill the males before they fertilize the females. Yes, folks...the most potent pot comes from 'horny females.'

The reason this amazing, very sophisticated, ET plant from the future is illegal has nothing to do with how it physically affects us…..

…POT IS ILLEGAL BECAUSE BILLIONAIRES WANT TO REMAIN BILLIONAIRES!

ps: I think the word 'DRUGS' should not be used as an umbrella-word that covers all chemical agents. Drugs have come to be known as something BAD. Are you aware there are LEGAL drugstores?! Yep, in every city. Unbelievable. Each so-called drug should be considered individually. Cannabis is a medicine and not a drug. We should DARE to speak the TRUTH no matter what the law is.

Also read Doug Yurchey's follow-up article, "Marijuana Conspiracy - the Sequel" here at the Illuminati News website.

ALTERNATIVE DRUGS & MEDICINE

Marijuana Conspiracy - the Sequel
by Doug Yurchey, May 29, 2007

Doug Yurchey
Doug Yurchey

n 2005, this writer penned 'The Marijuana Conspiracy - The Real Reason Hemp is Illegal' and it became (by far) my most popular article. Translated into Italian, French, Spanish, Russian, Swedish, Bulgarian and even Japanese, the responses were overwhelming. Its premise was 'marijuana' became illegal NOT because it was a danger to the mind and body. No, the real reason is that Big Business wants us to use petrochemicals and fossil fuels. They have little interest in NATURAL solutions. THEY have suppressed the truth concerning cannabis and purposely created the 'menace of marijuana.' Why? So they remain high profiteers while destroying our environment in the process.

Once people read the news of what has been kept from them, the truth, the reactions were outrage and disbelief at our federal authorities. Hemp used properly for all of its INDUSTRIAL purposes, as it was always meant to be utilized, could…

SAVE THE WORLD! We need not cut down another tree. We need not have to rely on Oil Companies anymore.

So many people wrote to me and said the suppression of hemp is ‘insane.’ Surely, governments know the potential of pot. They understand how cannabis hemp could solve the energy crisis; they know biodegradable plastics could be produced from the hemp plant; they realize it would be a safer, cleaner world filled with (inexpensive) quality products…if only we were federally given the green light to INDUSTRIALIZE HEMP! Governments are well aware of the MEDICINAL value for ‘weed.’ New cloths, papers and plastics would be super durable. They could easily create nutritional FOOD products for a starving world. BUT…when did a better mousetrap ever see the light of day?

Now, with soaring gas prices bleeding us for every dollar inflating all prices, we need a cheap (renewable) fuel and energy source more than ever. Imagine hemp gas. The price of it would not be sky high since we have a limitless supply of hempen energy. Federal (guarded) hemp fields could be farmed and directed to produce a cheap/clean gasoline. Hemp fibers and oil were utilized during WWII for rope-moorings, parachutes and lubrication. Why not use hemp gasoline NOW when we are at another critical point in time? Why does hemp technology remain clandestine?

Why is there a war in the Middle East over oil? Do you really think it’s a coincidence that George Bush is an oilman and remains steadfast against bringing our troops home? Hemp petroleum is the way to alleviate us from dependency on foreign oil. That is a wonderful dream, cannabis as the means to bring our troops home!

Email replies to the original article mainly asked WHY? Why don’t the authorities appreciate the profits to be made from hemp products? Money rules the world, so why don’t the industrialists make it legal? Tax hemp, regulate it, make it legal not for altruistic purposes but for their own high profits! The planet and its people would be much healthier. Why the HELL isn’t this done?

There are no easy answers. Generally, my reply to these readers scratching their befuddled heads was ‘secret societies.’ We are not educated on how the world really functions. We grow up watching movies and television where the good guys always win. We are trained to think that our government is good and out for the benefit of all. Films portray the heroes as CIA, FBI, NSA agents or obedient soldiers and police. We are given the false hope that governments care. Please do not be so naïve as to think this is true.

Power was never in the hands of the people. It is almost foolish to think that we, the general masses, control anything. Do you really think petitions work or writing to your congressional representative?

The truth is governments only care about themselves; we are their enemy and they need to control us (because we have the numbers) any way they can…with religion, bogus science, television, news, propaganda, commercials or via the abolition of MARIJUANA.

Instead of THEM industrializing hemp to save the planet…they have banned it. They have created a monster out of the very thing that is a precious gift to the world. NO, it does not make sense at all. However, it is understandable in the insane, Bizarro, backwards world that we live in. Apparently, any good thing is always destroyed. (Ever hear of Nikola Tesla?) Why? The answer must be POWER and CONTROL. The ‘haves’ do not want to give anything to the ‘have-nots.’ These are policies from the Dark Ages; they remain alive and well to this day. Research Secret Societies and you might understand.

They invented the monster of MARIJUANA. (‘If we do not have an enemy, they will create one’). They had to change the word since the world would never have been turned against HEMP. For thousands of years going back to the wisdom of the Egyptians, hemp was employed for the betterment of the human race. Enter ‘marihuana’ in the 1930s; just when farm machinery improved that would have made hemp the first ‘billion dollar crop!’

Have we grown up enough to see that they have pulled the wool over our eyes? We can no longer afford to be silent lambs. We have to speak up loud and strong and do what we can to spread the truth. What can we do? Share the information, wake people up to the true facts and suppressed knowledge concerning hemp. Eventually, when the pro-hemp people become the large majority, then, the world will change for the better. In the end, we will win.

A very important aspect of those email responses to my original article came in the form of personal stories. These testimonials answer the question: Does hemp help or hurt? Now, this writer could not please everyone on such a controversial subject. Not all the emails contained high praise. There were those anti-hemp fanatics spouting rhetoric from every commercial from the ‘Partnership of a Drug-Free America.’ Some stated it causes ‘lung cancer’ as they tried to educate me on the ‘toxins’ in marijuana.

Well, there are no toxins in hemp. I would love to tell Judge Judy, who is always yelling for someone to take a hair follicle test, the reason why THC stays in your system for so long. (And it’s ‘hemp’ not ‘marijuana’). If it were a toxin, a poison or some foreign substance, the body would immediate reject it and expel it. The body does NOT. It knows THC, the active ingredient of hemp, is GOOD. As mentioned before, there are essential fatty acids in hemp seeds. There are other sources in nature for these essential acids, but eating hemp seeds is a great source of (important) proteins. Our own bodies tell us the truth. Shouldn’t we listen to the truth within ourselves rather than the garbage broadcasted over television?

Seriously, the pro emails compared to the con emails ran 100 to one. For every one who blasted me, calling me names (these were from mainly young people)…there were about a HUNDRED administrators, educators, some scientists, activists and average people who related their (positive) private stories to me. Many claimed that normal medical procedures and doctors failed them miserably. The ONLY relief from their illnesses was illegal hemp. Here is a peek into some of the best and positive emails over the last two years. They are anonymous and edited, but they are real with the date I received them:

6/20/05 I was diagnosed with an AVM (Arterio Venous Malformation), a neuro-illness that causes me to have fits. On my second appointment with my neuro surgeon we went through the list of narcotics I was using at the time (marijuana). With that she gave me a sly look and said ‘off the record Mr. ____, this will actually reduce your blood pressure and can only be beneficial for your epilepsy. But I didn't tell you that.’

6/23/05 I took a college class in drug use, it was about street drugs, not pharmaceutical drugs. I learned that, at one time, marijuana was legal and was prescribed by MDs and dispensed by pharmacists for a variety of health problems - you know: HTN, insomnia, glaucoma, asthma, etc. I was told that the government decided to impose a $25 tax on MDs who prescribed it and a $35 tax on pharmacists who dispensed it. In protest, both groups elected to stop dealing with it. In response, Pres. Harriman included marijuana in his Narcotics Act of 1914: It was called the Harriman Act. They lumped it in as a class 4 narcotic meaning that there was no medical use for it, even though it's not a narcotic and no one had any indication that it was harmful in any way. In the class, we were shown pictures, taken during the early part of the century, of children smoking a joint…which, we were told, they could purchase for 5 cents, OTC, at any pharmacy, perfectly LEGAL - they were called ‘muggles.’

6/18/05 You didn't mention the most important reason why there is a continuing battle against legalization of marijuana, that is because it actually is used to treat sick people with pain successfully and with no serious side effects, something the legal drug industry hasn't accomplished with all their patented expensive crap they call medication. They don't want to loose their chokehold on the billions of dollars they make with this phony medication that could be replaced with an herb anyone can grow in their backyard.

6/18/05 I have been smoking pot since 1962 and I am now 83. I wish I had started when I was a teenager. Good for you.

6/18/05 I have a lot of respect for cannabis as I have used it medically for a spinal injury for several years recently, when all else failed, as doctors gave me narcotic drugs to relieve pain, and these drugs never worked and I was in such severe pain even with their drugs. Cannabis was the only pain reliever that worked. I have done a bit of research myself and found that cannabis is listed under a narcotic and this must be taken off the list that the United States has placed it as narcotic as opium, heroin, cocaine is totally ridiculous and bogus. I feel that if it was taken off the list and proved to the DEA, FDA, or whoever else…that it isn't a narcotic then we can get some headway on this wonderful, helpful plant. If it wasn't for cannabis I wouldn't have healed...The AMA is the worst, the most horrendous of all, because they encourage the doctors to prescribe drugs that lead to the deterioration of the body. Yes, it is a conspiracy, from the beginning, that the corporations don't want hemp to be grown. If they did, the logging industry would be wiped out. I live in southern Oregon and it is the biggest industry here…

6/19/05 I'm a 41 year old victim of Cystic Fibrosis, a genetic disease that kills people usually around 32 years old. I have the worst type of genetic mutation but still alive at the amazement of my Doctor. The reason? Maybe HEMP! I use it in a moderate way, around 2-3 times a week, usually eating it, and the benefit of that medicine is very good for me. Cystic Fibrosis kills people by pneumonia. An effect of hemp is drying mucus secretion so it kills bacteria by starvation. Even better: an antioxidant property of hemp that prevents lung tissue degradation. Another characteristic of Cystic Fibrosis is destruction of the digestive system, especially pancreas, stomach, liver and intestine. That is appetite inhibiting. I'm often eating because of holy hemp. It avoids me from losing too much weight and helps my defense against the constant lung infection. If my doctor knew the truth, he'd surely start hemp harvesting for sure!

6/20/05 I am a Disabled Veteran who spent 17 years taking the VA's Medication. I moved to Arizona; and a Pima Indian Medicine Man gave me an 'herb' to use as a tea to lower my blood pressure. Within 3 weeks it had gone from 210/146 to 130/70. Obviously, I quit the Government Medication and I now participate in the interesting hobby of home gardening of herbal cures for the ills of mankind! By the way, just so you don't think I'm 'a doper,' I served 8 years in the USAF, 2 years as a Palm Beach County FL Police Officer and then 17 years as a Paramedic. I have a Masters of Theology degree from Indiana Wesleyan University. Thanks for having the guts to publish THE TRUTH.

6/23/05 Doug, I also referred Montel Williams to your article about hemp 'marijuana' because he has multiple sclerosis and wants to legalize marijuana for medicinal purpose since it seems to help ms folks with pain. Since he has a TV talk show, I thought maybe he would give your article some publicity.

6/30/05 I am bipolar and when I smoke I am calmer then I am on my pills. I also have fibromyalgia and when in pain I grab my trusty friend Pot; it helps me function and eat. If I didn't have it, I would be dead years ago. Some days I can hardly make it down the stairs because of the ache and pains in my joints. Also, I suffer from severe migraines where I can't even open my eyes because I am light sensitive then my head pounds. I have been on many narcotics from vicodin to oxycottin and they all make me feel worse than smoking a damn joint. Only in America would they make a plant so beneficial to humankind, the most dangerous 'drug' in all the land. If I didn't go score I would be in pain and would never be able to walk out to get my mail. My fiancé gets upset since I can't work but she knows I can't help it. I want to work. I want to be the breadwinner of the family, but physically I cannot maintain standing for more than a few moments before I feel like I am passing out because of the pain. I went to numerous doctors and told them that pot helps me…all of them said since I live in Ohio, they cannot do anything for me besides narcotics…but I cannot smoke a joint…that is rubbish in my book.

7/31/05 I don't know if you have thought about this before, but after all the research I have done, it is my opinion that JFK was assassinated over hemp. He was a known user for his back pain, he fired Anslinger as soon as he got into office and it was said he was going to legalize it in his 2nd term. He never made it to his 2nd term. Maybe you can dig a bit more on your end and see what you come up with to support this theory of mine. It makes sense, don't you think? Keep educating people one day at a time…it's our only shot.

11/18/05 The actual truth that the oil giants do not want anyone to know is that most 'non producing' oil fields (for example, Derrick Island off Louisiana) have been found to have mysteriously refilled themselves after closing them down. This is because the vast majority of oil is not biotic but abiotic. The planet's crust produces billions of new gallons of crude annually and this is refilling the fields pumped out in almost every case. That is why Saudi Arabia just doubled the estimate of its known reserves over its predictions of 10 years ago. The wells keep refilling themselves due to planetary mechanics. Biotic oil is a discredited theory. There was never enough biomass in any prehistoric period to produce the amount of petroleum we have already consumed on this planet to date.

1/7/06 The most important health benefits come from smoking (or otherwise consuming) hemp/cannabis plant material which contains high levels of the active ingredients (THC, etc.) The higher the level of active ingredients, the more medicinal and beneficial it is. This is highly effective medicine, with no negative side effects, which is being denied to people in desperate need because of a pack of lies!

12/10/06 Weed is unprocessed and natural, as opposed to other processed drugs. I have eaten hemp seeds and have even had hemp oil and see the unspoken benefits from them even before I was planning to research the subject.

12/10/06 Maybe by the time I retire I can smoke a joint in peace, not fearing the Police and my government. But, other times it just seems hopeless, but I refuse to believe that money is the most powerful force in our Universe...there must be some room for truth.

12/16/06 I am a recreational therapist working for a state operated drug treatment center. If I were to speak in these terms, I doubt I would remain in favor of those above me.

2/2/07 As a medical marijuana user for many years, I would not be here without it. I was a vegetable on the prescribed medication and don't remember 2 whole years of my life while I was on morphine. Now I live a full and active life, running a business and bringing up a family. Is it too late to change views? I do hope not because it has so much to offer.

Above are some of the best comments from hundreds of replies. My article was far from perfect. I thank those more informed on plants than myself that told me there are other plants that physically reveal their sex such as the holly plant. Many criticized my belief that cannabis had an extraterrestrial origin. That conclusion came out of decades of studying our ancestors of prehistory. My studies have shown a completely new genesis for the human race where we have lost great knowledge. (It may have been advanced HUMANS that created Earthly animals and plants). Cannabis Sativa and all of its infinite and vital uses may be evidence that some great intelligence (with a plan) manufactured it. 'Chance' cannot account for its abundant purposes. To some people, the existence of hemp might be proof of the existence of GOD! My website link explores multiple controversies and truths that will never be aired on television. Check it out:

http://www.yurchey.com

Dare To Tell The Truth About Hemp

Writing about hemp, answering the emails and the entire Internet experience has taught me that there are all kinds of people. You cannot please everyone. What was 'brilliant' to one person was contemptible to another. People 'think' they know the truth, but the truth is they only know what they have been programmed to think. Very few people do independent research. That's too much work; they prefer sitting back, watching TV and letting others do their thinking for them. They do not QUESTION what has been handed them. But, if they did explore other possibilities outside of the norm...if they dreamed of a different world…and entertained the idea that we have been lied to by our leaders…maybe we would learn a little and come closer to the FREEDOM that we all rightfully deserve. A cleaner, safer planet will happen. It may not be soon, but it will happen in time…

Hemp

Dedicated to V for Vendetta and those with the courage to change the world for the better!
dugko@surfside.net

Explain Why There Is A Government Held Patent Acknowledging Marijuana's Medical Benefit
The federal government refuses to admit that marijuana has medical benefits when it comes to state policy. The feds also fight any attempts to reschedule marijuana. Currently, marijuana is a Schedule I substance, which is described as:
"Schedule I drugs, substances, or chemicals are defined as drugs with no currently accepted medical use and a high potential for abuse. Schedule I drugs are the most dangerous drugs of all the drug schedules with potentially severe psychological or physical dependence. "
No currently accepted medical use. If the feds fight state medical marijuana laws and programs, and keep marijuana a Schedule I substance, then why does it own a patent for medical marijuana? Why does the federal government distribute medical marijuana to four patients (as seen in the article photo)?
The US government obtained patent #6630507 in 2003 describing the medical benefits of marijuana, yet the people's government still schedules marijuana as having no medicinal value. The discrepancy needs to be explained, admitted, and changed.

From <http://www.theweedblog.com/why-does-the-federal-government-own-a-patent-for-medical-marijuana/>

PMC full text:
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Table 1
Effect of endocannabinoids on functions of different immune cells.
Immune cells
Functions affected
Receptor involved
Reference cited
T-lymphocytes
Proliferation; cell death by apoptosis; Th1/Th2 cytokine secretion, polarization; cell number
CB2
[46,47,55,56]
B-lymphocytes
Inhibition of antibody formation; Ig production; Ig isotype switching; proliferation; cell number
CB1 and CB2
[48,49,51]
Haematopoietic cell line
Cell growth
Non-CB1, CB2

[52,53]
Macrophages
Decreased Inflammatory mediators; antigen presentation; migration; phagocytosis; increased adhesion
CB2
[61–64]
Mast cells
Down modulate mast cell activation; decreased TNF-α; decreased mast cell-dependent angiogenesis
Non-CB1, CB2
CB1, CB2
[65]
[66,68]
Dendritic cells
Growth and maturation; apoptosis; recruitment during innate immune response
CB1, CB2
[72–74]
Natural killer cells and neutrophils
Cytolytic activity; chemokines; cytokines
Non-CB1, CB2
[75–78]
Cancer cells
Cell cycle arrest; apoptosis; growth inhibitor
CB1, CB2 receptor, TRPV1, lipid rafts
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Targeting the Endocannabinoid System to Treat Sepsis
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Abstract
The endocannabinoid system represents a potential therapeutic target in sepsis due to the presence of cannabinoid receptors (CB2) on immune cells. In this review we discuss how various targets within the endocannabinoid system can be manipulated to treat the immune consequences of sepsis. One of the targets outlined are the endocannabinoid receptors and modulation of their activity through pharmacological agonists and antagonists. Another therapeutic target covered in this review is the modulation of the endocannabinoid degradative enzyme's activity. Modulation of degradative enzyme activity can change the levels of endogenous cannabinoids thereby altering immune activity. Overall, activation of the CB2 receptors causes immunosuppression and can be beneficial during the hyperactivated immune state of sepsis, while suppression of the CB2 receptors may be beneficial during a hypoimmune septic state.
Key words: sepsis, endocannabinoid system, inflammation, immune modulation
Introduction
Sepsis is a complex immune disorder that can affect the function of almost all organ systems in the body. This disorder is characterised by a malfunctioning immune response to an infection that involves both pro-inflammatory and immunosuppressive mediators. This leads to severe damage and failure of vital organs, resulting in patient death (figures 1,2). Sepsis, septic shock, and systemic inflammatory response syndrome are the leading causes of mortality in surgical intensive care unit patients internationally. (1) Within the United States alone, the incidence of sepsis is around 750,000 annually, (2) resulting in more than 200,000 deaths. (3)
Older individuals with fragile immune systems are at a higher risk of developing sepsis after invasive surgical procedures. Concurrently, the proportion of our ageing population has been increasing steadily over the years with 8% of the population being over 60 years old in 1950, 11% in

2009, and predicted to be 22% in 2050. (4) As a result, the incidence of sepsis in North America is projected to rise along with the risk of patient mortality. (2) Unfortunately, current management of sepsis is limited to supportive care, and therapeutic intervention is mainly hindered by the complex pathophysiology and heterogeneous immuno-inflammatory nature of the disease. (5) Although supportive care is beneficial to septic patients, there are no specific therapeutic options available that target the immune system to eliminate septic related mortality. All of these facets have resulted in a significant cost to the heath care system. (2)

The current lack of viable therapeutic treatment options for sepsis underscores our insufficient understanding of this complex disease. The endocannabinoid system, a key regulator of essential physiological functions including the immune system, has recently emerged as a potential therapeutic target for sepsis treatment. The endocannabinoid system acquires its name from the plant Cannabis Sativa, which has been used medically to treat a variety of ailments, as well as recreationally for centuries. Cannabis Sativa contains more than 60 active phytocannabinoids with the primary phytocannabinoid Δ9-tetrahydrocannabinol (THC), (6) activating both endogenous endocannabinoid receptors. (7)

The endocannabinoid system and sepsis

The endogenous endocannabinoid signalling system can be thought of as consisting of three main elements that mediate its signalling. The first element consists of the endocannabinoids or bioactive lipid signalling elements. The second element is the endocannabinoid receptors that bind endocannabinoids. Finally, the third element encompasses proteins and transporters that mediate biosynthesis, transport and degradation of endocannabinoids. (8-11)

To-date, two endocannabinoid receptors have been identified: cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors. These receptors are members of the Class A Rhodopsin-like family of G-protein coupled receptors and, therefore, contain seven transmembrane domains. Both CB1 and CB2, preferentially couple to heterotrimeric Gi/o proteins and adenyl cyclases, but may also couple to other classes of G proteins. (8,9). CB1 receptors were the first cannabinoid receptor to be molecularly cloned. (12) CB1 receptors are expressed ubiquitously in the body, but are most abundant in the central nervous system (CNS), where they modulate neurotransmitter release. (13) In keeping with this, the behavioural effects of cannabinoid ligands are associated with the CB1 receptor in the CNS. CB2 receptors were discovered shortly after the identification of CB1 receptors, (14) and their expression is largely restricted to immune cells such as lymphocytes, macrophages, and neutrophils. (15) The expression of CB2 receptors in immune cells in humans is as follows; B cells > Natural Killer Cell > Monocytes > Polymorphonuclear Neutrophils > CD8 Leukocytes > CD4 Leukocytes. (16)

Cells involved in the inflammatory response can be broadly distinguished into two categories: cells present in tissues, and cells that travel in the blood. The cells present in the tissues comprise of mast cells, endothelial cells and tissue macrophages, while the inflammatory cells within the blood stream are platelets and leukocytes. Leukocytes are further subdivided into polymorphonuclear (multi-lobed nuclei) cells and mononuclear cells. The polymorphonuclear cells are subdivided into neutrophils, basophils, and eosinophils, while the mononuclear cells are divided into monocytes and lymphocytes. (17) The inflammatory cells present in the tissue are activated and secrete inflammatory mediators when physically damaged or chemically stimulated, prompting localization of leukocytes to the site of damage. Cells of the immune system release cytokines that are protein mediators to either increase or decrease the inflammatory response. The cytokine sub-classification includes interleukins (ILs), chemokines, interferons, colony stimulating factors, tumour necrosis factors (TNFs), and growth factors. (17) TNF-α and IL-1 are the main pro-inflammatory cytokines associated with the LPS induced hyper-immune response of sepsis, while IL-10 is associated with the subsequent immune suppression of sepsis immunopathology. (18)

Since the discovery of the cannabinoid receptors, extensive research has been focused on elucidating their functions; (19) we now know that the endocannabinoid system is involved in an array of diverse functions like immune modulation, cancer, pain processing, anxiety, depression, and neuroprotection. (20) This diverse influence of the endocannabinoid system has made it an attractive potential drug target for the treatment of a wide array of human disease. Multiple pharmacological approaches have also been devised to manipulate the endocannabinoid system ranging from development of synthetic cannabinoid receptor ligands, to compounds that alter the biosynthesis or degradation of endogenous endocannabinoid ligands. (21,22) Due to the predominance of CB2 receptor expression on immune cells, it is likely that they play a role in the function of the immune system as well as the inflammatory response. (23) Therefore modulation of these receptors and their ligands for therapeutic benefit will be the main focus of this review.

Two endogenous endocannabinoids have been most extensively studied. (24) They are: arachidonoylethanolamide (AEA; previously known as anandamide), (25) and 2-arachidonoylglycerol (2-AG) (figure 3). (26) Both AEA and 2-AG have the ability to activate both CB1 and CB2 receptors. AEA and 2-AG are released locally and have a short duration of action; the concentrations of these endocannabinoids are tightly regulated to avoid excessive stimulation. (10,22) Extracellular levels of these two endocannabinoids are strictly regulated by the system to avoid excess stimulation. The endocannabinoids are rapidly eliminated from the extracellular environment through cellular reuptake followed by enzyme hydrolysis. (10) Two main enzymes are responsible for the cytosolic degradation of the endocannabinoids. Fatty acid amide hydrolase (FAAH) is a membrane bound serine hydrolase enzyme that is responsible for the hydrolysis of AEA as well as a large number of endogenous fatty acid amides (figure 3). (27,28) Monoacylglycerol lipase (MAGL) is the primary enzyme responsible for the degradation of 2-AG (figure 3), (28) however FAAH and a few other enzymes also have the ability to degrade 2-AG.

Therapeutic Targets

Effective treatment of sepsis requires the proper identification and understanding of the patient's septic state. Current understanding now indicates that sepsis immunopathogenesis starts with an early hyper-inflammatory response followed by a later anti-inflammatory response (figure 1). (28,29) Due to this biphasic pathology of sepsis, immunosuppression through the endocannabinoid system may not always be a beneficial therapy. The early hyper-inflammatory response is characterised by an uncontrolled over activation of the innate immune response to pathogens. During this phase, an excessive amount of pro-inflammatory mediators are released from immune cells to mobilize a defensive response to the pathogens. Unfortunately, this exaggerated response causes damage to the host's tissue, leading to hypoperfusion and eventual organ failure. (28) During this phase of sepsis, a reduction in the release of pro-inflammatory cytokines and mediators will be helpful to minimize tissue damage. Modulation of the endocannabinoid system, particularly CB2, (23) to dampen down the inflammatory response during this phase of sepsis may be a beneficial therapy. Alternatively, patients in later stages of sepsis have a suppressed immune system, a state initiated by their own immune system as a mechanism to counteract the preceding hyper-immune response. Unfortunately, tissue damage and organ failure can continue to occur in this phase from the invading pathogens left in the system. As a result during this phase, modulation of the endocannabinoid system to upregulate the inflammatory response may help to reduce mortality by eliminating invading pathogens.

Endocannabinoid receptors

Modulation of the endocannabinoid system at the signalling elements, receptors or degradative enzymes, may provide a potential therapeutic benefit during inflammation and sepsis. One viable option is to directly target cannabinoid receptors using receptor selective drugs, where modulation of specific receptors can result in therapeutic benefit. (30) Previous studies have indicated that activation of presynaptic CB1 receptors on autonomic nerves and vascular walls contribute to the hypotension associated with septic shock. (31) This result is not conclusive however because when CB1 knockout mice were subject to acute endotoxemia, acute hypotension was still observed. (32) Studies in our own lab have found that inhibition of the CB1 receptor, using the antagonist AM281, protected microcirculation of the gut and iris during acute endotoxemia. (33,34)

Manipulation of CB2 receptors may have good utility in sepsis treatment given the localized expression of these receptors to macrophages, neutrophils and lymphocytes. Activation of CB2 receptors is generally associated with an antinflammatory effect, with a resultant decrease in pro-inflammatory cytokines and a reduction in macrophage and neutrophil recruitment. (35) However, research on the modulation of these receptors has also provided some contradictory results. (36,37)

A study by Tschöp et al. modulated the CB2 receptor in a systemic septic model induced through cecal ligation and puncture (CLP) in mice. (36) They observed reduced survival time of CB2 receptor knockout mice compared to wild type mice during systemic sepsis. Furthermore, the CB2 receptor knockout mice showed increased neutrophil recruitment and decreased neutrophil activation at the site of infection, leading to additional inflammatory damage. To observe CB2 receptor involvement in inflammatory mediation, they also used a highly specific CB2 receptor agonist called

GP1a in wild type mice and observed increased mean survival time, decreased neutrophil recruitment, and increased neutrophil activation at the site of infection. Finally, the activity of a MAP kinase p38 known to regulate neutrophil function and priming was shown to be increased at the site of infection and associated with CB2 activation. (36) Altogether, these results indicated that activation of the CB2 receptor during systemic inflammation can help reduce mortality and tissue damage.

Another study by Csoka et al. also used a systemic inflammation model of sepsis through cecal ligation and puncture. (37) However, in the findings of this study they showed that CB2 receptor knockout mice have significantly lower mortality rates as well as decreased number of bacteria within the blood, but similar levels of bacteria in the peritoneal lavage fluid when compared to wild type mice. They also measured serum levels of IL-10, an anti-inflammatory cytokine produce by macrophages, because an overproduction of this cytokine can impair immune defense to bacterial pathogens. They observed lower levels of IL-10 in both the blood serum and peritoneal lavage of knockout mice compared to wild type. All of these results indicated that CB2 receptor activation during systemic sepsis increased IL-10 overproduction causing an increase in bacterial invasion into the blood, ultimately resulting in increased mortality rates. (37)

Multiple factors may help explain the inconsistency reported in studies of manipulating the CB2 receptor during a septic state. (36,37) A possible explanation could be the severity of sepsis, where certain factors may play a crucial role in determining the role of the endocannabinoid receptors. Studies from our own lab indicate that in a more chronic stage of sepsis, inhibition of CB2 receptors reduced leukocyte activation and restored capillary perfusion. (34) Furthermore the stage of sepsis immunopathogenesis could be different between the mice used in these two studies. (36,37) For example, activation of the CB2 receptors during the immunosuppressed stage of sepsis can cause increased mortality, possibly explaining the results seen by Csoka and colleagues. Taken together, all these results indicate that early in disease progression of sepsis, activation of the CB2 receptors may be beneficial given that it increases activation of neutrophils at the site of infection, while preventing additional neutrophil recruitment. However, in the latter stages of sepsis, an inhibition of CB2 receptors may be beneficial in order to prevent additional inflammatory damage incurred by preventing the activation of more leukocytes. Additional research needs to be conducted in order to strengthen these conclusions.

Degradative Enzymes

Apart from manipulating the receptors themselves, regulation of endocannabinoid levels by targeting their enzymatic degradation may provide potential therapeutic benefit during sepsis. Although significant work has been carried out to-date on some of these endocannabinoid degradative enzymes, (10,27,38,39) since their discovery, to our knowledge, only a few studies have investigated the role of specific enzymes in sepsis. (40,41) With respect to FAAH, the key hydrolase for AEA degradation, cytokines such as IL-4 & IL-10 have been shown to influence FAAH activity by increasing its activity, (40) and septic patients show a down-regulation of FAAH expression, leading to increased available AEA levels. (40,41) This is consistent with the elevation in AEA levels seen in experimental models in which FAAH activity is lost. (42) Increased AEA levels may be beneficial during the initial stages of sepsis to stimulate CB2 receptors and help combat the exaggerated immune response. However, sustained elevated AEA levels can also be detrimental in the latter stages of sepsis by further suppressing the immune system, causing a heightened vulnerability to the invading pathogens. To our knowledge, the role of MAGL inhibition on sepsis and inflammation has not yet been documented, but chronic loss of MAGL function is associated with increased 2-AG levels, CB1 desensitization and hyperalgesia. (43)

Our lab has explored the therapeutic potential of FAAH inhibition in experimental sepsis (endotoxemia). Rats were treated with a selective FAAH inhibitor, URB597, at different dosages, prior to the induction of sepsis. Furthermore, a separate group of rats were also treated with a selective CB2 antagonist along with URB597 and LPS, to verify the role of CB2 receptor activation. Our results showed that an LPS challenge significantly increased leukocyte adhesion to the endothelial lining in the collecting and post-capillary venules of the intestinal submucosa. Furthermore, capillary perfusion, measured through functional capillary density (FCD), was significantly decreased in both the circular and longitudinal muscle layer of the gut, as well as the intestinal mucosal villi during the LPS challenge. The group pretreated with URB597 showed a prevention of the increased leukocyte adhesion promoted by LPS. URB597 pre-treatment also prevented the LPS induced reduction in FCD, however the effect was only seen at the higher dose of URB597 and only in the circular muscle layer of the gut. The use of the CB2 receptor antagonist, AM630, along with LPS and URB597 reversed the actions of the FAAH inhibitor on leukocyte adhesion for both collecting and post-capillary venules. This result indicates that the protective effects of URB597 were mediated by CB2 activation. However, AM630 administration did not have any significant impact on the effects of URB597 on the FCD either in the mucosa or intestinal muscle layers. (44)

Conclusion

The endocannabinoid system modulates the immune response in experimental sepsis. Manipulating the endocannabinoid system may have potential therapeutic benefit in clinical sepsis where immune and inflammatory dysfunction can be detrimental. Multiple targets exist within the endocannabinoid system, e.g. the system can be targeted at the level of receptors by administration of synthetic compounds, similar to the endocannabinoids, which either increase or inhibit receptor activation to provide the desired therapeutic effect. Alternatively, the endogenous enzymes that degrade endocannabinoids or cannabinoid-like lipids can also be targeted in order to manipulate the levels of endocannabinoids. Proper identification of the septic stage is crucial to determine the adequate therapeutic response that will be most beneficial. Due to the biphasic nature of sepsis immunopathology, immune suppression through endocannabinoid modulation can help mitigate the hyper-immune response during the early septic state, while immune activation may be beneficial in later stages.

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Figure 1. Immune response in sepsis and potential therapeutic endocannabinoid modulation.

Figure 2. The sepsis cascade.

Figure 3. Production and degradation of endocannabinoids AEA & 2-AG. Phospholipid precursors are modified by N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and Diacylglycerol lipase (DAGL) to produce AEA and 2-AG respectively. MAGL degrades 2-AG into arachidonic acid and glycerol, while FAAH degrades AEA into arachidonic acid and ethanolamine.

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Artificial Neural Networks and the Study of the
Psychoactivity of Cannabinoid Compounds

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Cannabinoid compounds have widely been employed because of its medicinal and psychotropic properties. These compounds are isolated from Cannabis sativa (or marijuana) and are used in several medical treatments, such as glaucoma, nausea associated to chemotherapy, pain and many other situations. More recently, its use as appetite stimulant has been indicated in patients with cachexia or AIDS. In this work, the influence of several molecular descriptors on the psychoactivity of 50 cannabinoid compounds is analyzed aiming one obtain a model able to predict the psychoactivity of new cannabinoids. For this purpose, initially, the selection of descriptors was carried out using the Fisher's weight, the correlation matrix among the calculated variables and principal component analysis. From these analyses, the following descriptors have been considered more relevant: E_{LUMO} (energy of the lowest unoccupied molecular orbital), Log P (logarithm of the partition coefficient), VC4 (volume of the substituent at the C4 position) and LP1 (Lovasz–Pelikan index, a molecular branching index). To follow, two neural network models were used to construct a more adequate model for classifying new cannabinoid compounds. The first model employed was multi-layer perceptrons, with algorithm back-propagation, and the second model used was the Kohonen network. The results obtained from both networks were compared and showed that both techniques presented a high percentage of correctness to discriminate psychoactive and psychoinactive compounds. However, the Kohonen network was superior to multi-layer perceptrons.
Key words:
cannabinoids, neural network models, psychoactivity
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Hashish and marijuana, two preparations derived from *Cannabis sativa* L., have been used since long past because of its medicinal and psychoactive properties (1). The large popularity of marijuana among recreational drug users is because of its mind-altering effects. However, this drug also has considerable therapeutic potential documented since the fourth century BC (2). Marijuana has been employed as medical treatment including glaucoma, hypertension, nausea associated to chemotherapy, pain, migraine and many other conditions. More recently its use as an appetite stimulant has

been indicated in patients with cachexia or wasting disease such as AIDS (3,4).

Despite of the isolation of Δ9-tetrahydrocannabinol (Δ9-THC) as the major psychoactive component of *Cannabis sativa* L. in the 1960s, the biological targets of Δ9-THC were only discovered in the late 1980s, allowing the accomplishment of suitable cannabinoid receptor binding assays and the characterization of the biochemistry and the molecular biology of cannabinoid receptors, which led to two subtypes of G-protein-coupled receptors named CB1 (5) and CB2 (6).

The immunosuppressive properties of plant-derived cannabinoids have been widely investigated (7–9). Δ9-THC, the most extensively investigated cannabinoid, exhibits a broad range of immunomodulatory activity (10).

Nowadays, (quantitative) structure–activity relationship or (Q)SAR studies have been proved to be helpful in the understanding of the influence of molecular properties on the biological activity presented by several classes of compounds (11–14). It is well accepted that physicochemical properties and structural attributes of chemical compounds govern their biological activities (biological response) (15).

The search for quantitative relationships between the chemical structure (i.e. physicochemical, structural and conformational properties) and the biological response is the subject of quantitative structure–activity relationship (QSAR) studies. These relationships will hopefully help to understand and explain the driving forces behind the drug action, ultimately supporting the development of new compounds exhibiting desirable biological properties. One of the most important characteristics of QSAR models is their predictive power (15,16).

In the past 25 years, several statistical methods have broadened the arsenal of tools that can be applied to (Q)SAR studies (15). A certain number of computational techniques have been found useful for the establishment of these relationships such as multiple linear regression, principal component analysis (PCA) and partial least squares. Recently, there is also a growing interest in the application of artificial neural networks (ANN) in the field of (Q)SAR (17).

Artificial neural networks (ANN)

Finding SARs is essentially a pattern recognition process, and, historically, (Q)SAR models have been developed using linear methods such as linear discriminant analysis. However, several non-linear (Q)SAR methods have been proposed in recent years. Artificial neural networks are increasingly being used in (Q)SAR studies (18), and this technique is frequently used for data analysis with increasing interest in chemistry and related fields of research since 1986 (19,20).

Artificial neural networks has application in compound classification, modeling of SARs, identification of potential drug targets and localization of structural and functional features of biopolymers (17). Besides, ANN techniques have been applied to the fields of robotics, pattern identification, psychology, physics, computer science, biology and many others. Artificial neural networks are also used for the modeling of several systems in a wide range of applications such as animal science (21), simulation of

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C nuclear mag-
netic resonance spectra (22), problem of drug
/
non-drug
classification (23), predicting the stability of RNA
/
DNA hybrid
duplexes (24), prediction of biological targets (25) and as well as predicting physicochemical properties from the perspective of pharmaceutical research (26).

The main advantages of ANNs are the following: their flexibility enables them to discover more complex relationships in data than traditional statistical model; capability of extracting essential process information from data and the property of generalization, i.e. they can correctly process information that only broadly resembles the original training data. Perhaps, the most important advantage of all is that ANN allows going directly from factual data to the models without any human subjective interference (27). The ANN involves a training step in which the model parameters are determined from known data (training set), a validation step in which the model performance is established and a prediction step in which the property of novel compounds is predicted by the optimized neuron (28).

A very important characteristic of neural networks is that they are adaptive, i.e. they can take data and learn from them. This ability does not depend upon the prior knowledge of rules. With only a few exceptions, ANNs are able to generate non-linear models, and they are capable of learning complex interactions among the input variables in a system even when they are difficult to find and describe.

As it is not possible to know
a priori
which molecular properties
are most relevant to the problem at hand, ANN, like other modeling methods, is often used in conjunction with optimization techniques for feature selection (29). In this work, we have used two neural networks, multi-layer perceptrons (MLP) and Kohonen, to study the psychoactivity of 50 cannabinoid compounds employing the variables selected previously (30) and to demonstrate the performance of the two ANN procedures for the series of compounds studied.

Methodology

Compounds studied

Cannabinoid molecules have a typical structure as shown in Figure 1 (the numbering system used in this work is also shown). The chemical structure of each cannabinoid molecule studied, along with its indication of biological activity, is displayed in Figure 2. The 50 cannabinoid compounds studied here were classified into two classes: actives and inactives, based on the effects caused on rhesus monkeys when the compounds were intravenously injected (31–35).

Multi-layer perceptrons

The multi-layer perceptrons (also termed as feed-forward neural network because all of the data information flows in one direction) are composed of multiple processing units called neurons (or nodes) arranged in several different layers (36). The first layer consists of the input variables, and the last layer consists of the output vari-

8
7
6
10
10
9
6
O
4
10
4
3
2
1
11
OH
C
5
H
11
a
a

a
b
5
A
B
Figure 1:
General structure and
numbering used for the cannabi-
noid compounds studied.

ables representing the output class. Intermediate layers called hidden layer receive the entire input pattern that is modified by the passage through the weighted connections. The hidden layer provides the internal representation of neural pathways. Training means a search process for the optimized set of weight values, which can minimize the squared error between the estimated and
O
CH
3
OH
C
5
H
11
C
H
3
C
H
3
Ac1 (
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
Ac2 (
Δ
8
-THC)
O
CH
2
OH
OH
C
5
H
11
C
H
3
C
H
3
Ac3 (11-hidroxy-
Δ
9
-THC)
O
OH
C
5
H

11
C
H
3
C
H
3
CH
2
OH
Ac4 (11-hidroxy-
Δ
8
-THC)
O
OAc
C
5
H
11
C
H
3
C
H
3
CH
3
Ac5 (
Δ
9
-THC acetate)
O
OAc
C
5
H
11
C
H
3
C
H
3
CH
3
Ac6 (
Δ
8
-THC acetate)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
CH
3
Ac7 (2-methyl-
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3

C
H
3
CH
3
C
2
H
5
Ac8 (2-ethyl-
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
CH
3
Ac9 (2-methyl-
Δ
8
-THC)
O
C
5
H
11
C
H
3
C
H
3
CH
3
OH
C
2
H
5
Ac10 (2-ethyl-
Δ
8
-THC)
O
CH
3
OH
C
5
H
11
H
2
C
C
H
3
OH
Ac11 (12-hidroxy-
Δ
9
-
THC)
O
OH
C
5

H
11
C
H
3
C
H
3
CH
3
O
H
Ac12 (8-hidroxy-
Δ
9
-THC)
O
OH
C
3
H
7
C
H
3
C
H
3
CH
3
Ac13 (
Δ
9
-
Tetrahydrocannabivarin)
O
OH
C
6
H
13
C
H
3
C
H
3
CH
3
Ac14 (hexyl homolog of
Δ
9
-THC)
O
OH
C
6
H
13
C
H
3
C
H
3
CH
3
Ac15 (hexyl homolog of
Δ
8
-THC)
O
OH
CH(CH
3
)C
4
H
9

C
H
3
C
H
3
CH
3
Ac16 (1
′
-methylpentyl
homolog of
Δ
9
-THC)
O
CH(CH
3
)C
4
H
9
C
H
3
C
H
3
CH
3
OH
Ac17 (1
′
- methylpentyl
homolog of
Δ
8
-THC)
O
OH
CH(CH
3
)
C
H
3
C
H
3
CH
3
CH(CH
3
)C
5
H
11
Ac18 (1
′
,2
′
-
dimethylheptyl homolog
of
Δ
9
-THC)
O
OH
CH(CH
3
)
C
H
3
C
H
3
CH

3
CH(CH
3
)C
5
H
11
Ac19 (1
′
,2
′
-
dimethylheptyl homolog
of
Δ
8
-THC)
O
OH
CH(CH
3
)
C
H
3
C
H
3
CH
3
CH(CH
3
)C
3
H
7
Ac20 (1
′
,2
′
-
dimethylpentyl homolog
of
Δ
8
-THC)
O
OH
C
H
3
C
H
3
CH
3
CH
2
CH(OH)
(CH
2
)
2
CH
3
Ac21 (2
′
-hidroxy-
Δ
8
-
THC)
O
OH
C
H
3
C
H

3
CH
3
(CH
2
)
2
CH(OH)
CH
2
CH
3
Ac22 (3
′
-hidroxy-
Δ
8
-
THC)
O
OH
C
H
3
C
H
3
CH
3
(CH
2
)
3
CH(OH)
CH
3
Ac22 (4
′
-
hidroxy-
Δ
8
-
THC)
O
OH
C
H
3
C
H
3
CH
3
(CH
2
)
5
OH
Ac24 (5
′
-hidroxy-
Δ
8
-
THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CHO

Ac25 (11-oxo-
Δ
8
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
2
NHCH
3
Ac26 (11-methylamine-
Δ
8
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
Ac27 (9-nor-
Δ
8
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
Ac28 (
Δ
10a
-THC)
Figure 2:
Molecular structure
and indication of psychoactivity
presented by the cannabinoid compounds studied.

experimental data of units in the output layer (37). There are many training algorithms for MLP neural networks, and the network can be trained using different algorithms such as back propagation, conjugate gradient descent, quasi-Newton, Levenberg-Marquardt, quick propagation, delta-bar-delta, etc. (38). Back propagation learning algorithm is widely used in multi-layer feed forward networks, and the calculations begin in the output layer and progress backward through the network to the input layer. The training algorithm is used to adjust the network's weights and thresholds to minimize this error. Because a target value is compared to the output value,

the learning is called supervised (39,40).

According to previous study (30), several molecular descriptors were calculated for a set containing 26 cannabinoid compounds, and a selection of these descriptors was carried out using the Fisher's weight, the correlation matrix among the calculated variables and PCA. From these analyses, it was possible to reduce the high dimensionality of data, making the data interpretation and visualization

O
OH
CH
3
C
H
3
C
H
3
CH
3
(CH)N(OH)
Ic19 (3-methyl-4-oxim-
Δ
10a
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
C
2
H
5
CH
3
Ic20 (4-ethyl-
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
OH
Ic21 (10-hidroxy-
Δ
8
-THC)
O
OH
C
6
H
13
C
H
3
C
H
3
CH
3

Ac29 (hexyl homolog of
Δ
10a
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
COOH
Ic1 (2-carboxy-
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
COOH
Ic2 (2-carboxy-
Δ
8
-THC)
O
CH
3
OH
C
5
H
11
C
H
3
C
H
3
COOCH
3
Ic3 (2-carbomethoxy-
Δ
9
-
THC)
O
OH
C
5
H
11
C
H
3
C
H
3
COOCH
3
CH
3

Ic4 (2-carbomethoxy-
Δ
8
-
THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
COCH
3
Ic5 (2-acetyl-
Δ
8
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
COCH
3
Ic6 (4-acetyl-
Δ
8
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
COOCH
3
CH
3
Ic7 (4-carbomethoxy-
Δ
8
-
THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH

3
COOH
Ic8 (4-carboxy-
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
CH
3
Ic9 (4-methyl-
Δ
9
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
C
2
H
5
CH
3
Ic10 (4-ethyl-
Δ
8
-THC)
O
OH
C
5
H
11
C
H
3
C
H
3
CH
3
CH
3
Ic11 (4-methyl-
Δ
8
-THC)
O
OAc
C
5
H
11
C
H
3
C
H

3
COOCH
3
Ic12 (11-carbomethoxy-
Δ
8
-THC acetate)
O
OAc
C
5
H
11
C
H
3
C
H
3
CH
3
O
Ic13 (8-oxo-
Δ
9
-THC
acetate)
O
H
O
OAc
C
5
H
11
C
H
3
C
H
3
CH
3
Ic14 (7-hidroxy-
Δ
8
-THC
acetate)
O
OAc
C
5
H
11
C
H
3
C
H
3
OAc
CH
3
Ic15 (4-acetoxy-
Δ
8
-THC
acetate)
O
O
C
5
H
11
C
H
3
C
H

3
CH
3
O
Ic16 (
Δ
8
-THC quinone)
O
C
5
H
11
C
H
3
C
H
3
CH
3
OMe
Ic17 (methyl-ether-
Δ
8
-THC)
O
OH
CH
3
C
H
3
C
H
3
CH
3
CN
Ic18 (3-methyl-4-cian-
Δ
10a
-THC)
Figure 2:
Continued

easier than the analysis using the original data set. So, the most relevant descriptors selected and considered responsible for the discrimination between the active and inactive cannabinoid compounds were as follows: E
LUMO
(energy of the lowest unoccupied molecular orbital), Log P (logarithm of the partition coefficient), VC4 (volume of the substituent at the C4 position) and LP1 (Lovasz–Pelikan index, a molecular branching index) (30).

The data set (50 cannabinoid compounds and the four descriptors selected previously) was used to test the performance of the ANN techniques. Initially, the data set was divided into five sets of 10 molecules each. Using leave-one-out cross-validation (41,42), each one of these data sets was used as a test set with the other four sets serving as training sets. Therefore, each one of the 50 cannabinoid molecules appeared only once over all of the test sets.

The descriptors employed in our ANN analyses were those selected previously (E
LUMO
, Log P, VC4 and LP1) (30). Table 1 displays the main parameters employed in the ANN approaches used in this work.

The four descriptors selected (E
LUMO
, Log P, VC4 and LP1) correspond to the number of neurons into input layer, and the input patterns (training set) are the 50 cannabinoid compounds used to train the neural network. The output layer indicates the two classes of cannabinoid compounds studied (psychoactive and psychoinactive). It is important to notice that a cross-validated process was

employed with the aim to assess the predictive capability of the ANNs used. So, some subsets of compounds have been chosen, and the class, constituted by these compounds, has been predicted by ANNs. The learning algorithm used (back-propagation) implements a gradient search over the error response surface for the set of weights that minimizes the sum of the squared error between the actual and target values.

Kohonen network (self-organizing map)

Kohonen introduced an ANN that he called the self-organizing network. Learning in a Kohonen network is unsupervised, i.e., the property to be investigated is not used in the training process (43). Its main goal is to perform a non-linear mapping from a high-dimensional variable space to a low-dimensional (usually 2D) target space, so that the distance and proximity relations between the samples, – or, in a single word, the topology – are preserved (44). In Kohonen network or self-organizing map (SOM), each neuron is represented by a weight, which is a vector of same dimension as the input signal. The input is passed to each neuron in the network, and the neuron whose weight vector is the closest to the input signal is declared the 'winner'. Not only the winning neuron has its weights adjusted, but also the neurons in its neighborhood. The extent of adjustment depends, however, on the topological distance to the winning neuron – the closer a neuron is to the central neuron, the larger is the adjustment of its weights. The objects of the training set are iteratively fed to the map, the weights corrected, and the training is stopped when a predefined number of cycles are attained. A trained SOM will reveal similarities in the objects of a data set in the sense that similar objects are mapped into the same or closely adjacent neurons (45,46).

The advantage of the SOM, compared with some other projection methods, is that the algorithm is very simple, straightforward to implement and fast to compute. This multivariate tool was successfully applied to chemical analysis problems using atomic emission spectroscopy and atomic X-ray spectrometry (47) and to diagnose calibration problems in atomic absorption spectroscopy (48). Besides these applications, its potentialities have been shown in the polymer degradation (49), soil classification using near infrared spectroscopy (50), modeling toxicity (51), design of novel trypanocidal quinones compounds (52), among others (53).

The SOM toolbox, developed by the Laboratory of Computer and Information Science, Helsinki University of Technology, Finland, is available free of charge as a Matlab toolbox at the Web site
a
. The computation environment used was Matlab version 6.5, developed by Mathworks
b
.

Results and Discussion

Multi-layer perceptrons

In this work, 24 new cannabinoid compounds, with similar molecular structure, were added in the initial data set [the 26 cannabinoid compounds studied previously (30)]. So, the final data set now has 50 cannabinoid compounds and four molecular descriptors. The values of E_{LUMO}, Log P, VC4 and LP1 for the 50 cannabinoid compounds studied here are presented in Table 2.

Table 3 displays the results obtained from the ANN analysis. From Table 3, the results can be analyzed as follows: the two classes of compounds were defined by two codes, i.e. the psychoactive compounds were labeled with code (1 0) and the psychoinactive ones with the code (0 1). After the training step, each compound had a code defined by the ANN method. Comparing the real code (real class) and the ANN code of the cannabinoid compounds studied, we can analyze the quality of the ANN results.

According to Table 3, only two compounds (Ac21 and Ac25) presented different codes to those corresponding to the real class, i.e. the compounds Ac21 and Ac25 (real code = 1–0) had the codes given by the ANN technique equal (0.340–0.659) for compound Ac21 and (0.288–0.711) for compound Ac25, indicating that these compounds were classified incorrectly. In any case, the results obtained from the ANN methodology presented high percentage of correct information (96%), and this indicates that the descriptors selected (E_{LUMO}, Log P, VC4 and LP1) can be considered very important when one wants to design new cannabinoid

compounds.

Table 1:
Main characteristics of artificial neural networks used in this work

Number of neurons into input layer	4
Number of neurons into output layer	2
Algorithm of learning	Back-propagation
Number of input patterns used	50

Kohonen network (self-organizing map)

To improve the data interpretation, SOM was built with 50 cannabinoid compounds. The SOM map was built using the four descriptors as described earlier (E_{LUMO}, Log P, VC4 and LP1). In Figure 3, also named Labels, the cannabinoids correspond to the mapping of the 50 samples from a space of X-dimensional onto a bi-dimensional region, and each sample is represented by one neuron (the winner neuron). From Figure 3, we can see that SOM methodology indicated a good separation of each group into two regions: one for active compounds and the other for inactive ones.

From the SOM map obtained (Figure 3) with four descriptors selected, we can see that the compounds Ac21 and Ac25 are

Table 2:
Values of the four descriptors selected for the 50 cannabinoid compounds studied

Compound	Activity	E_{LUMO} (eV)	Log P	VC4 (^{3})	LP1
Ac1	Active	0.411	5.35	71.70	2.551
Ac2	Active	0.398	5.35	71.70	2.551
Ac3	Active	0.385	4.34	71.70	2.552
Ac4	Active	0.373	4.34	71.70	2.552
Ac5	Active	0.291	5.13	71.70	2.558
Ac6	Active	0.254	5.13		

71.70
2.558
Ac7
Active
0.412
5.81
71.70
2.564
Ac8
Active
0.414
6.21
71.70
2.568
Ac9
Active
0.398
5.81
71.70
2.564
Ac10
Active
0.407
6.21
71.70
2.568
Ac11
Active
0.257
4.56
71.70
2.559
Ac12
Active
0.348
4.43
71.70
2.560
Ac13
Active
0.412
4.55
71.70
2.550
Ac14
Active
0.411
5.74
71.70
2.551
Ac15
Active
0.397
5.74
71.70
2.551
Ac16
Active
0.427
5.68
71.70
2.554
Ac17
Active
0.415
5.68
71.70
2.554
Ac18
Active
0.425
6.80
71.70
2.556
Ac19
Active
0.412
6.80
71.70

2.556
Ac20
Active
0.414
6.01
71.70
2.556
Ac21
Active
0.206
4.29
71.70
2.551
Ac22
Active
0.446
4.29
71.70
2.551
Ac23
Active
0.478
4.22
71.70
2.551
Ac24
Active
0.369
4.20
71.70
2.551
Ac25
Active
0.036
4.17
71.70
2.552
Ac26
Active
0.389
4.40
71.70
2.553
Ac27
Active
0.392
5.19
71.70
2.543
Ac28
Active
)
0.043
5.24
71.70
2.551
Ac29
Active
)
0.043
5.64
71.70
2.551
Ic1
Inactive
)
0.328
5.04
71.70
2.573
Ic2
Inactive
)
0.382
5.04
71.70
2.573
Ic3
Inactive

)
0.163
5.08
71.70
2.574
Ic4
Inactive
)
0.244
5.08
71.70
2.574
Ic5
Inactive
)
0.090
4.65
71.70
2.573
Ic6
Inactive
0.107
4.65
214.97
2.580
Ic7
Inactive
0.002
5.08
240.14
2.582
Ic8
Inactive
)
0.040
5.04
180.51
2.580
Ic9
Inactive
0.425
5.81
151.99
2.569
Ic10
Inactive
0.423
6.21
211.48
2.574
Ic11
Inactive
0.413
5.81
152.14
2.569
Ic12
Inactive
)
0.018
4.39
71.70
2.563
Ic13
Inactive
)
0.261
4.74
71.70
2.566
Ic14
Inactive
0.176
4.26
71.70
2.575
Ic15
Inactive
0.065

4.63
234.24
2.583
Ic16
Inactive
)
1.430
4.10
92.73
2.569
Ic17
Inactive
)
0.483
3.62
155.26
2.570
Ic18
Inactive
)
0.169
3.99
201.32
2.571
Ic19
Inactive
0.224
4.51
71.70
2.571
Ic20
Inactive
0.433
6.21
211.59
2.574
Ic21
Inactive
0.224
4.51
71.70
2.571
Table 3:
Results obtained using the artificial neural network technique
Compound
Activity
Real class
Predicted class
Ac1
Active
1
0
0.999
0.000
Ac2
Active
1
0
0.999
0.000
Ac3
Active
1
0
0.999
0.000
Ac4
Active
1
0
0.999
0.000
Ac5
Active
1
0
0.999
0.000

Ac6
Active
1
0
0.921
0.078
Ac7
Active
1
0
1.000
0.000
Ac8
Active
1
0
1.000
0.000
Ac9
Active
1
0
1.000
0.000
Ac10
Active
1
0
1.000
0.000
Ac11
Active
1
0
0.820
0.179
Ac12
Active
1
0
0.999
0.000
Ac13
Active
1
0
0.999
0.000
Ac14
Active
1
0
1.000
0.000
Ac15
Active
1
0
1.000
0.000
Ac16
Active
1
0
0.999
0.000
Ac17
Active
1
0
0.999
0.000
Ac18
Active
1
0
1.000
0.000
Ac19

Active
1
0
1.000
0.000
Ac20
Active
1
0
1.000
0.000
Ac21
Active
1
0
0.340
0.659
Ac22
Active
1
0
0.999
0.000
Ac23
Active
1
0
0.999
0.000
Ac24
Active
1
0
0.999
0.000
Ac25
Active
1
0
0.288
0.711
Ac26
Active
1
0
0.999
0.000
Ac27
Active
1
0
0.999
0.000
Ac28
Active
1
0
0.937
0.062
Ac29
Active
1
0
0.991
0.008
Ic1
Inactive
0
1
0.004
0.995
Ic2
Inactive
0
1
0.009
0.990
Ic3
Inactive

0
1
0.005
0.994
Ic4
Inactive
0
1
0.002
0.997
Ic5
Inactive
0
1
0.019
0.980
Ic6
Inactive
0
1
0.000
1.000
Ic7
Inactive
0
1
0.000
1.000
Ic8
Inactive
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0.000
1.000
Ic9
Inactive
0
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0.023
0.976
Ic10
Inactive
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0.999
Ic11
Inactive
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1
0.012
0.987
Ic12
Inactive
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0.204
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Ic14
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0
1
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0.677
Ic15
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Ic16
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0.989
Ic17
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Ic18
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1.000
Ic19
Inactive
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1
0.391
0.608
Ic20
Inactive
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1
0.000
0.999
Ic21
Inactive
0
1
0.391
0.608
Percentage
of correct information
96%
Psychoactivity of Cannabinoid Compounds
Chem Biol Drug Des
2010; 75: 632–640
637
defined in a region of the map containing active compounds, and these compounds are defined by an unique neuron with compound Ac12 and thus are considered as one subgroup. Therefore, according to Figure 3, we can see that compounds Ac21 and Ac25 were correctly classified as active, differing to that one generated by MLP neural networks. This result is very interesting as it agrees with the previous study (30).

Figure 4 shows the component maps, each one corresponding to the compounds as well as those variables determined before. The value of the variables can be read on the scale (right side of each map). Considering both Figures 3 and 4, the value of the variable for each sample can be recognized. The position occupied by a sample in the Label map (Figure 3) corresponds to the same position in the component maps (Figure 4).

From Figure 4, we can see that for a cannabinoid compound to present psychoinactivity, VC4 and LP1 need to have high values (i.e. the compound need to present a bulky substituent at the C4 position and several branchings in the overall structure), whereas the other variables (E_{LUMO} and Log P) need to have low values.

Therefore, to design new psychoinactive cannabinoid compounds, it is necessary to consider that the candidate molecule should have high values for VC4 and LP1, along with low values for E_{LUMO} and Log P. In chemical words, the four variables (E_{LUMO}, Log P, VC4 and LP1) responsible for the discrimination between psychoactive and psychoinactive cannabinoid compounds belong to four different classes: electronic (E_{LUMO}), hydrophobic (Log P), steric (VC4) and topological (LP1), indicating that the interaction between the cannabinoid compounds studied and the biological receptor can occur as a result of electronic, hydrophobic, steric and topological features of these compounds. In fact, from this

study, we can conclude that the model obtained here by using two different ANN techniques gave reliable results and certainly can be used to predict the psychoactivity of new cannabinoid compounds with similar molecular structure to those studied in this work.

Figure 3:
Compound mapping onto a bi-dimensional space using four descriptors selected. All compounds were assigned to one of two groups according to their activities. Grey and white represent active and inactive groups, respectively.

Figure 4:
Compound component maps, where each map shows the calculated descriptors. The scales are related to the respective calculated descriptors displayed in Table 2.

Conclusions

A set of 50 cannabinoid compounds was studied by using two ANN techniques (MLP and Kohonen) with the aim to verify the importance of the four previously selected descriptors (E_{LUMO}, Log P, VC4 and LP1) to classify new cannabinoid compounds. From the ANN results, we can conclude that the set of variables used is able to discriminate between psychoactive and psychoinactive cannabinoid compounds, and the two ANN techniques used in this work were able to give us the most relevant characteristics when the psychoactivity of cannabinoid compounds is studied, as it classified the compounds studied with a high level of reliability. It is important to notice that the Kohonen network supplied better outcomes when compared to the MLP ones. This demonstrates that ANNs are really useful in this case, where the set of cannabinoid compounds studied has a high structural diversity. Further, the model obtained by using the ANN techniques provided reliable results, and they can be used to predict the psychoactivity of new cannabinoid compounds with similar molecular structure to those studied.

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Notes
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The cannabinoid system and immune modulation

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Abstract

Studies on the effects of marijuana smoking have evolved into the discovery and description of the endocannabinoid system. To date, this system is composed of two receptors, CB1 and CB2, and endogenous ligands including anandamide, 2-arachidonoyl glycerol, and others. CB1 receptors and ligands are found in the brain as well as immune and other peripheral tissues. Conversely, CB2 receptors and ligands are found primarily in the periphery, especially in immune cells. Cannabinoid receptors are G protein-coupled receptors, and they have been linked to signaling pathways and gene activities in common with this receptor family. In addition, cannabinoids have been shown to modulate a variety of immune cell functions in humans and animals and more recently, have been shown to modulate T helper cell development, chemotaxis, and tumor development. Many of these drug effects occur through cannabinoid receptor signaling mechanisms and the modulation of cytokines and other gene products. It appears the immunocannabinoid system is involved in regulating the brain-immune axis and might be exploited in future therapies for chronic diseases and immune deficiency.

• marijuana
• Th1 cells
• chemotaxis
• tumor
• G protein
• CB1

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INTRODUCTION

The normal immune system is modulated and regulated by foreign antigens that induce the production and secretion of a plethora of proteins from immune and nonimmune cells. These proteins include cytokines such as interleukin (IL)-2, chemokines such as monocyte chemotactic protein-1 [1], and surface receptors and other membrane proteins. In addition to these primary immune modulators, other humoral factors and metabolic products also modulate immunity. For example, prostaglandins [2], steroids [3], and histamine [4] are known to exert powerful effects on the quantitative and qualitative aspects of immunity. In addition, mediators of the brain-immune axis such as catecholamines [5], endogenous opioids [6], and cannabinoids [7 , 8] are also capable of modulating immune function. It is reasonable to speculate that these latter factors serve as secondary modulators, which when mobilized coincident with or shortly after first-line immune modulators such as lymphokines, increase or decrease immune activity. These secondary mediators are relatively simple in structure and therefore have been synthesized. This, along with the fact that the pharmacology of these agents in many cases has been thoroughly studied, makes these compounds good candidates for use in immunotherapy.

The medicinal uses of marijuana were described centuries ago for diseases such as asthma, migraine, pain, convulsions, and anxiety (reviewed in ref. [9]). More recently, emphasis has been placed on marijuana's putative, beneficial effects on appetite, glaucoma, spasticity in multiple sclerosis, pain, and inflammation [10]. Recent experimental evidence supports marijuana's therapeutic potential in some of these maladies [11]. The active plant ingredients in marijuana belong to the C21-cannabinoid compounds including the primary psychoactive compound, Δ9-tetrahydrocannabinol (THC). This cannabinoid along with others such as Δ8-THC, cannabidiol, and cannabinol, as well as chemical analogs, have been extensively studied over the years for their biological and therapeutic properties [8]. Some of the properties of these agents have included effects on immunity ranging from suppression of resistance to infection to enhancement of IL-1 production by macrophages. These early studies about the immunomodulating effects of these drugs have been the subject of previous overviews [7 , 12 13 14 15 16 17 18] and will not be reviewed here. Instead, we will briefly summarize the general features of the cannabinoid system and review recent findings on the structure and function of the cannabinoid system components in the immune system. For convenience, we will refer to this as the "immunocannabinoid" system.

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CANNABINOID SYSTEM

Marijuana cannabinoids, analogs, and endocannabinoids

The flowering tops and leaves of the marijuana plant contain many chemicals including the C21, tricyclic, cannabinoids, the most widely studied of these is THC, which is also the most potent in terms of psychoactivity (for review of structures, see ref. [8]). Cannabinoids are lipophilic and therefore difficult to work with; however, several were isolated in the 1940s, and by 1964, THC was isolated and synthesized [19]. Elucidating the structure allowed for the subsequent synthesis and testing of active analogs with varying cannabimimetic potencies leading to the pharmacological demonstration of cannabinoid receptors [9 , 20]. Of the many analogs synthesized, several have been used extensively over the years. One widely studied analog is the dimethylheptyl derivative of 11-hydroxy-Δ8-THC (HU210), which has a much higher affinity for cannabinoid receptors than THC (ref. [21];Table 1). In addition to the tricyclic cannabinoids, bicyclic analogs, such as CP55,940 [29], and aminoalkylindoles, such as R-(+)-WIN55,212 [30], have also been synthesized with cannabimimetic activity and with affinities higher than THC (Table 1) . All of these agents have been widely used in examining the biology of the cannabinoid system and as can be seen from Table 1 , these agents are relatively nonselective for cannabinoid receptors in that their Kis are similar for both. More recently, CB2-selective agonists have been synthesized, such as JWH-015, which displays a higher affinity for CB2 than for CB1 (ref. [31]; Table 1).

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Table 1.
Binding Affinities of Cannabinoid Receptor Ligands

Besides the classical agonists, such as THC, and nonclassical agonists, such as HU210, other ligand groups have been described. One of these, the so-called endocannabinoid group, is chemically eicosanoid in structure, and its members are derived from free arachidonic acid (ref. [32]; Fig. 1). The first of this group was isolated from pig brain and was determined to be an arachidonyl ethanolamide. This compound was named anandamide [33] and was shown in some systems to be relatively nonselective for either of the cannabinoid receptors (Table 1) . The existence of this endogenous ligand had been predicted by the discovery of cannabinoid receptors, and its demonstration established the existence of the endocannabinoid system of receptors and ligands. More recently, several other endocannabinoids have been isolated from tissues, such as 2-AG [26] and 2-AGE [34], the latter of which appears to be relatively selective for CB1. Anandamide and 2-AG are produced in brain as well as spleen and other peripheral tissues [35 , 36] and catabolized by the membrane-bound serine hydrolase, fatty acid amide hydrolase [37], and monoacylglycerol lipase [36], respectively. In addition to binding to cannabinoid receptors, anandamide also binds to vanilloid receptors, which are ligand-gated cation channels sensitive to

capsaicin and related analogs [38].

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Figure 1.
The endocannabinoid system and cell function. An influx of Ca2+ activates phospholipases (PL), which drive the conversion of membrane arachidonic acid (AA) to endocannabinoids such as anandamide (ANA), 2-arachidonoylglycerol (2-AG), and 2-arachidonylglyceryl ether (2-AGE). The endocannabinoids can be taken up and metabolized by fatty acid amide hydrolase (FAAH) or can bind to CB1 or CB2. These are G protein-coupled receptors (GPCR) capable of signaling through G protein subunits, Gα, -β, and -γ, leading to a modulation of nitric oxide (NO) and adenylyl cyclase (AC). In neurons, this signaling can lead to a change in K+ and Ca2+ currents and secretion of transmitters such as γ-aminobutyric acid (GABA). In addition, CB1 and CB2 signaling can lead to the activation of many other factors, including PLC, protein kinase C (PKC), nuclear factor-κB (NF-κB), extracellular signal-regulated protein kinase (ERK), focal adhesion kinase (FAK), steroid receptor coactivator (Src), mitogen-activated protein kinase (MAPK), and MAPK kinase (MEK).
Another group of cannabinoid receptor ligands is the diarylpyrazole group, which has antagonist/inverse agonist activity when tested in a variety of tissues. The first of these described is the CB1-selective compound, SR141716A [23], which has potent antagonist activity for the CB1 receptor. The second described is the CB2-selective compound, SR144528, which has potent CB2 antagonism (ref. [28]; Table 1). Since the description of these two, other antagonists have been reported, and these have recently been reviewed [8].
Cannabinoid receptors
Pharmacological evidence initially supported the existence of cannabinoid receptors of high affinity in brain tissue and with specificity sufficient to distinguish different enantiomers of cannabinoid analogs [20 , 39]. Subsequent to this, the first receptor, CB1, was cloned from a rat brain cDNA library using a probe derived from the sequence of bovine substance-K receptor [40]. The translated sequence of the gene yielded a protein of 473 amino acids and the structure of a 7-transmembrane, GPCR. Since then, homologues of CB1 have been cloned from humans [41] and mice [42], displaying 97–99% amino acid sequence identity from one species to the other. CB1 mRNAs are expressed in brain, especially in the basal ganglia, as well as several tissues in the periphery including immune tissue (see below). Polymorphisms in the human gene have been reported, including a G-to-A silent mutation at position 1359 in the coding exon and an (AAT)n repeat polymorphism outside of the exon [43]. The significance of these changes in terms of gene function and disease is not clear at this time. The CB1 coding sequence is contained in a single exon, but other cDNA segments have been reported, such as a 5′-untranslated first-exon and a large 3′-untranslated sequence. The second receptor, CB2, was cloned from a cDNA library from the promyelocytic, human cell line, HL60 [44]. The protein encoded by this gene was reported to contain only 360 amino acids and showed only 44% identity with the human CB1 receptor. The mouse [45] and rat [46] CB2 genes also have been cloned and encode proteins of 347 and 410 amino acids, respectively. The variation in length among these proteins occurs in the carboxy end with the rat protein containing an extended carboxy terminus [46]. Amino acid identity of CB2 from species to species is lower than for CB1, and the mRNAs for CB2 are found in peripheral tissues, such as spleen, but not in brain and tissue, such as heart.
Function of cannabinoid system
Cannabinoid receptors are GPCR, and very early on, it was recognized that they were linked to inhibition of adenylyl cyclase (ref. [20]; Fig. 1). In addition, GPCR are known to be linked to a variety of other second messengers and signaling components [47 48 49], and ligation of CB1 and CB2 has been shown to not only suppress adenylyl cyclase but also to activate it [50], suggesting these receptors activate factors other than Gαi [51]. In addition to adenylyl cyclase, cannabinoid receptor ligation has been shown to activate PLC [52], which in turn, might activate diacylglycerol and inositol triphosphatase, increasing intracellular calcium and PKC. Ligation also has been shown to inhibit Ca2+ currents and activate K currents [53 , 54] and to increase NO [55] synthesis (Fig. 1) . Cannabinoid receptor ligation also has been shown to lead to the activation of transcription factors such as members of the MAPK [56] and NF-κB [57 , 58] families. From this and other evidence, it is clear that signaling through cannabinoid receptors and other GPCR involves numerous second messengers and signaling factors and that a variety of cellular and genetic activities are regulated by these kinds of receptors, including neuronal excitability [59] and IL-12 gene expression [49].
Cannabinoid receptors and ligands are found in the brain and peripheral tissues, and our current understanding of its function, at least in the brain, is beginning to take shape. The role of endocannabinoids and cannabinoid receptors in modulation of neurotransmitter release recently has been reviewed [59 60 61]. Several reports in 2001 showed that CB1 is located on presynaptic nerve terminals, that endocannabinoids are released from depolarized postsynaptic neurons, and that CB1 agonists and antagonists can modulate presynaptic neurotransmitter release [62 63 64]. Stimulation of the CB1 receptors was shown to suppress the release of neurotransmitters, such as GABA, thus allowing the cannabinoid system to turn down the activity of the GABA terminal in a retrograde manner back across the synapse. In the case of GABA release in hippocampal cells, the cannabinoid system appears to mediate depolarization-induced suppression of inhibition and may account for the inhibitory effects of cannabinoid ligands on memory and movement [60].
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IMMUNE MODULATION
Cellular effects
Prior to the discovery of the cannabinoid receptors and the endocannabinoids, numerous studies examined the effects of marijuana smoking or THC on immune cell function. These studies have been extensively reviewed over the years [7 , 12 13 14 15 16 17 18] and support several conclusions. The first of these is that moderate marijuana smoking has little in the way of acute effects on systemic immunity but may have significant suppressive effects on the function of immune cells directly exposed to the smoke. For example, marijuana smoking was associated with suppressing the antimicrobial activity, cytokine production, and cytokine responsiveness of pulmonary alveolar macrophages [65 , 66]. However, regarding heavy or chronic use, sufficient studies have not been done to conclude that intense drug exposure is not without consequence on immune cell function. In fact, several studies suggest that long-term use in humans may be associated with increased incidence of infection [67 , 68], squamous cell carcinoma of the head and neck [69 , 70], and modulation of cannabinoid receptors in immune cells [71]; in nonhuman primates, heavy use may be associated with suppression of lymphocyte [72] and alveolar macrophage [73] function. Another conclusion from early studies is that treatment of mice and rats with THC or treatment of immune cell cultures from humans and rodents has, for the most part, a suppressive effect on T and B cells, natural killer (NK) cells, and macrophages. Various functions from cytotoxic T cell killing to antibody production by B cells to phagocytosis and killing by macrophages have been studied. Most functions were suppressed by drug treatment, and the effective drug concentrations in vitro were in the μM range. As this concentration range was higher than the reported receptor affinities, it was concluded by many that nonspecific effects of these lipophilic drugs on cell membranes primarily mediated the effects of cannabinoids on immune cell function. However, since the cloning of the receptors, it is now recognized that immune cells do express cannabinoid receptors and therefore, the modulating effects of cannabinoids are being reexamined in the context of cannabinoid receptor function. In addition, questions are being asked concerning the possible role of the immunocannabinoid system of receptors and ligands in the normal regulation of immunity.
Cannabinoid receptor expression in immune cells
By Northern blotting, the initial report on the cloning of CB1 failed to detect the expression of CB1 mRNA in tissues outside of the brain [40]. However, the cloning of the human gene demonstrated the message in testis tissue, but again, other tissues, including immune system tissues, were negative [41]. In 1992, Kaminski et al. [74] reported the demonstration of CB1 mRNA in mouse splenocytes using the more sensitive method of reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of receptors by these cells was also supported by pharmacological studies using cannabinoid agonist effects on in vitro antibody production by the splenocytes. Several agonists, such as THC, CP55,940, and HU210, suppressed antibody production in a dose-dependent manner when added to sheep red blood cell-stimulated splenocyte cultures. However, the affinity

and efficacy of these agonists were lower with spleen cells than reported for behavioral studies and studies involving neural tissue, suggesting a lower density of surface receptors and variation from neural tissue in receptor coupling and affinity in immune cells. Also, as immune cells express CB2 receptors in addition to CB1 (see below), it is not clear whether CB1 or CB2 was being stimulated in these studies. Subsequent studies showed that CB1 mRNA levels varied among cell subpopulations. In human peripheral blood cells, message was reported to be most abundant in B cells, and the rank order was B cells > NK cells > polymorphonuclear neutrophils (PMNs) > CD8 cells > monocytes > CD4 cells [75]. The level of message also varied among different leukocyte cell lines; Jurkat cells were negative, and THP1 cells were highly positive [75]. CB1 message levels also varied in mouse immune subpopulations, and consistent with human cells, splenic B cells expressed more message than macrophages or T cells [76 77 78]. In addition to mouse splenocytes, CB1 message has been detected in rat brain microglia cells [79] and in human peripheral blood cell-derived dendritic cells (DC) [80]. We also have observed that CB1 and CB2 mRNAs are expressed in bone marrow-derived DC from mice (unpublished results).
Antibodies to CB1 have been produced and used to examine the expression of CB1 protein on immune cells. Rabbit polyclonal antiserum to a CB1–glutathione-S-transferase (GST) fusion protein demonstrated several immunoreactive proteins in membrane preparations from Jurkat and a CB1-positive neuroblastoma cell line. Proteins of 45, 60, 70, and 87 kDa were consistent with CB1 expression [81]. Similarly, a 58-kDa protein was demonstrated in membranes from rat microglia cells using an antiserum to CB1–GST fusion proteins [79], and proteins of 64 and 83 kDa were demonstrated in human DC [80]. We have prepared polyclonal rabbit antiserum to the 14 terminal amino acids of CB1 conjugated to keyhole limpet hemocyanin (KLH) and used this to analyze cell membrane proteins from mouse splenocyte immune cell subpopulations. Figure 2 shows the antiserum detected a major band at 80 kDa in splenocyte subpopulations with B cells and macrophages showing more protein than T/NK cells. In addition, the neuroblastoma cell line, N18TG2, showed several bands, and the predominant one was at 67 kDa, suggesting a variation in immunoreactive protein size between brain cells and immune cells. We also tested several commercial polyclonal antisera prepared to CB1 peptides. Figure 3A and 3B , shows these antibodies demonstrate major bands at 65 kDa (Company A) and 75 kDa (Company B) and that these bands are also the major ones demonstrated in the neuroblastoma cell line. Curiously, all three antisera (Fig. 3A 3B 3C) demonstrate analogous protein bands in spleen cell preparations from CB1 homozygous knockout mice provided by Dr. Andreas Zimmer and co-workers [82]. This could result from several factors. The first is that other proteins expressed in mouse immune and neural tissue share epitopes with the CB1 protein. A search of the mouse genome using the website <http://www.ensembl.org/> and the 14 amino acids of the amino terminal end of the mouse CB1 protein shows scores of identical matches of five amino acids or less in the translated portion of the genome, suggesting that these CB1 epitopes are shared by many proteins of the mouse. A second possibility stems from the fact that in the knockout mice, the CB1 gene is disrupted with the neor cassette in the middle of the single-coding exon, leaving short 5′ and 3′ portions of this exon intact [82]. In fact, mRNA containing the 5′ end of the coding exon up to position 93 (accession #U22948) can be detected by RT-PCR in splenocytes from these mice at a higher level than in wild-type mice (unpublished results). It is possible, therefore, that fusion proteins containing amino and carboxy fragments of CB1 plus the neor insert are expressed in these mice and are being detected by the antisera. We are currently testing antiserum to epitopes coded by the deleted portion of the CB1 gene to see whether it can detect proteins of comparable size in immune tissue from knockout and wild-type mice and N18 cells. It is possible that this antiserum will detect comparable bands in only wild-type and N18 cells, suggesting the possibility of expression of CB1 fusion proteins in knockout mice. At this time, the specificity of available anti-CB1 antibodies remains in question.

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Figure 2.
Western blotting of membrane proteins from splenocytes using anti-CB1 antibody. Affinity-purified, polyclonal antiserum to CB1 peptide (MKSILDGLADTTFR-C) was used (160 ng/ml) to probe membrane proteins from splenocyte subpopulations and N18TG2 neuroblastoma cells. Lane 1, N18 cells; lane 2, whole splenocytes; lane 3, nonadherent splenocytes; lane 4, adherent splenocytes; lane 5, T cells; lane 6, B cells.

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Figure 3.
Analysis of CB1 by Western blotting of splenocyte membrane proteins from wild-type and knockout mice. (A) Membrane proteins probed with anti-CB1 antibody from Company A: lane 1, N18TG2 neuroblastoma cells; lane 2, wild-type splenocytes; lane 3, wild-type splenocytes; lane 4, homozygous knockout splenocytes. (B) Membrane proteins probed with anti-CB1 antibody from Company B: lane 1, N18 cells; lane 2, wild-type splenocytes; lane 3, wild-type splenocytes; lane 4, homozygous knockout splenocytes. (C) Membrane proteins probed with antibody prepared as in Figure 2 : lane 1, N18 cells; lane 2, homozygous knockout splenocytes; lane 3, heterozygous knockout splenocytes; and lane 4, wild-type splenocytes. USF, University of South Florida.
Unlike CB1, CB2 was cloned from a leukocyte cell line rather than brain tissue [44]. CB2 transcripts were detected in HL60 cells and rat spleen but not in other tissues such as thymus and brain. mRNA for the receptor was also detected by RT-PCR in macrophages from rat spleen but not in T cells [44]. CB2 mRNA expression was extended to human tissues, wherein by Northern blotting, it was demonstrated in only spleen and peripheral blood mononuclear cells (PBMC) and not in other tissues including brain; however, by RT-PCR, it was demonstrated in immune tissues including PBMC, thymus, spleen, and tonsils [83]. When examining PBMC subpopulations, CB2 message was in the rank order of B cells > NK cells > monocytes > PMNs > T cells. These and other studies suggested that CB2 mRNA was expressed primarily in immune cells but not in brain and that the message was most abundant in B cells [46 , 77 , 83 , 84]. Rodent peritoneal macrophages, cell lines, and microglia cells were also shown to be positive for CB2 mRNA when examined by mutational RT-PCR [85]. As in other studies, the CB2 message was more abundant than CB1 and was readily expressed in cortical primary microglia.
As with CB1, antibodies also have been used to examine the expression of CB2 protein on immune cells. Polyclonal antiserum to a C-terminal peptide of CB2 was used for immunofluorescence studies in human tonsillar tissue [83]. Immunoreactive tissue was highest in germinal centers, suggesting B cells or antigen-presenting cells (APCs) were expressing the receptor. Polyclonal antiserum produced to amino acids 320–336 of the mouse protein conjugated to KLH was used to examine CB2 protein expression in homogenates of cultured rat peritoneal macrophages and brain microglia [85]. The main immunoreactive protein in peritoneal macrophages was ~40 kDa, and in microglia preparations, the protein was 27 kDa. The reason for the relatively small size of the microglia protein was not clear but was suggested to be a result of a degradation product of the predicted 40-kDa protein or a variant form unique to microglia [85]. In addition to these studies, commercial antibodies were used to analyze CB2 protein in rat spleen and human blood-derived DC [80]. In these samples, immunoreactive bands of 39, 47, and 59 kDa were observed, although the reason for the multiple bands was not clear. As with anti-CB1 antibodies, the specificity and reliability still remain in question.
Modulation of cannabinoid receptor expression by cell activation
Immune cells frequently express new gene products when stimulated with antigens and other bioactive substances. This also appears to be the case with cannabinoid receptors. The initial suggestion of this came with the cloning of CB2, wherein it was shown that CB2 mRNA was increased in HL60 cells by treatment with agents such as phorbol myristate acetate (PMA) [44]. At basal levels, HL60 cells were negative for CB2 mRNA by Northern blotting but became positive by 24 h following 12-O-tetradecanoylphorbol 13-acetate treatment. Since this report, stimuli, ranging from lipopolysaccharide (LPS) to anti-CD40 antibodies (Fig. 4), have been shown to increase CB2 expression. For example, the level of CB2 mRNA and protein in human tonsillar B cells [86] and mRNA in mouse splenocytes [84] was increased following activation by anti-CD40 antibody, and CB2 expression was increased by IFN-γ in mouse macrophages and microglia [85]. In addition, we recently showed that chronic marijuana smoking

increased the CB2 mRNA in PBMC [71], suggesting that drug exposure may cause a change in receptor expression in humans as has been reported with CB1 in animal models [87]. Stimulation of immune cells also leads to a suppression of CB2 expression (Fig. 4) . For example, the differentiation of B cells in tonsils was accompanied by decreased expression of CB2 mRNA and protein in centroblasts as opposed to virgin B cells [86], and the stimulation by LPS of mouse splenocytes, macrophages, rat microglia, and human DC resulted in decreased expression of CB2 message [80 , 84 , 85]. In addition, TGF-β treatment of cultured human peripheral blood lymphocytes led to a decrease in CB2 protein with increasing concentration of the cytokine [88].

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Figure 4.
CB1 and CB2 expression is increased or decreased following cell activation. Stimuli reported to increase CB1 are LPS, anti-CD40 antibody, PHA, and marijuana use, and agents suppressing CB1 are LPS, PMA/ionomycin (Io), and anti-CD3 antibody. Stimuli reported to increase CB2 are PMA, anti-CD40 antibody, interferon-γ (IFN-γ), and marijuana use, and agents suppressing CB2 are LPS, differentiation, and transforming growth factor-β (TGF-β). PHA, Phytohemagglutinin.
CB1 expression is also modulated in immune cells by various stimuli (Fig. 4) . Initially, it was reported that PHA treatment in Jurkat cells [81] and LPS treatment of the mouse macrophage cell line, RAW 264.7 [89], induced an increase in CB1 message within several hours following treatment. However, studies with other cell types such as rat microglia [85] and human DC [80] showed that LPS treatment decreased rather than increased mRNA. The results with mouse splenocytes were even more complex with PMA/ionomycin or anti-CD3 antibody treatment, causing a decrease in CB1 message, and anti-CD40 treatment caused an increase [78]. It also has been reported that chronic marijuana smoking led to increased CB1 mRNA in PBMC, suggesting that drug use not only increased CB2 (see above) but also CB1 receptor expression [71]. From these studies, it is likely that the level of CB1 and CB2 receptors is modulated like many other immune gene products to varying degrees in various immune cell subpopulations. Although a few general themes are emerging in this regard, additional studies are needed using purified cells and detection reagents to define receptor expression in the immunocannabinoid system.

Cannabinoid receptor signaling in immune cells

A number of studies have defined various signaling events associated with CB1 and CB2 (see Function of cannabinoid system section). Many of these results were obtained in receptor-transfected cell systems or primary cells other than immune cells. However, a few reports have examined signaling events in immune cells in response to cannabinoid treatment. For example, intracellular calcium mobilization was studied in concanavalin A (Con A)-treated mouse thymocytes using fluorescent indicators and drug treatment with THC [90]. Cytosolic-free calcium was increased by Con A treatment, and this effect was suppressed by treatment with THC. The suppression of cytosolic calcium mobilization was a result of a drug-induced inhibition of extracellular influx as well as inhibition of calcium release from intracellular stores [90]. THC was also shown to suppress forskolin-stimulated adenylyl cyclase activity in mouse spleen cells [91] and purified splenic T cells [77]. The suppression of adenylyl cyclase by THC and CP55,940 was shown to be attenuated by treatment with pertussis toxin, suggesting that the cannabinoids were acting through Gi proteins [92].
Members of the NF-κB family of proteins also have been shown to be modulated in immune cells. The NK-like cell line, NKB61A2 [93], when treated with THC, was shown to increase the transcription of the IL-2Rα gene and increase the nuclear level of NF-κB protein [58]. However, in the macrophage cell line, RAW 264.7, THC was shown to inhibit NF-κB activity in response to treatment with LPS [57], suggesting that the type of cannabinoid effect on this family of proteins may be cell type- and stimulus-dependent. Several other immune cell lines have been tested for signaling modulation following cannabinoid treatment. The HL60 cell line was treated with CP55,940 and was shown to express higher levels of MAPK and Krox-24 gene activation [56], suggesting these signaling properties were increased by the drugs. In addition, EL4.IL2 mouse lymphoma cells treated with cannabinol or THC showed a decrease in PKA activity, activated protein-1, and NF-activated T cells binding [94 , 95]; furthermore, treatment of splenocytes with cannabinol decreased ERK–MAPK activity [96]. From these results, it appears that cannabinoid treatment of immune cells leads to modulation of various cannabinoid receptor-signaling pathways. This fact, coupled with immune modulation effects of these agents, suggests that cannabinoids may modulate immune cell function through CB1 and CB2 signaling mechanisms.

Modulation of T helper (Th) cells

Th cells are powerful regulators of cell-mediated (Th1) and humoral (Th2) adaptive immunity. Regulation of the development of these cells and the link between innate and adaptive immunity are areas of intense investigation [97] to better understand how infectious agents and other environmental factors affect the processes fundamental to activating host immunity. It is now clear that in addition to microbial products, drugs and endogenous factors regulate the development of Th1 and Th2 cells (Fig. 5). For example, prostaglandins increase Th2 activity and decrease Th1 activity [2]. In addition, glucocorticoids [98], morphine [99], and certain chemokines [49] are reported to suppress Th1 immunity, and adrenergic agents such as norepinephrine increase Th1 immunity [100].

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Figure 5.
Drugs and endogenous factors regulate the development of Th cells. Naïve CD4 Th (Th0) receive immune signals and in addition, receive signals from drugs and endogenous factors in the selective differentiation to Th1 or Th2 cells. Th1 development is decreased (↓) by prostaglandins, glucocorticoids, morphine, chemokines, and cannabinoids and is increased (↑) by norepinephrine. In contrast, Th2 development is decreased (↓) by norepinephrine and increased (↑) by prostaglandins, glucocorticoids, morphine, chemokines, and cannabinoids.
Cannabinoids were observed to suppress cell-mediated immunity to Legionella pneumophila infection when injected into mice shortly before infection. In addition, immunoglobulin G1 antibodies to Legionella were also increased in drug-treated mice, suggesting that THC caused, besides a decrease in cell-mediated immunity, an increase in Th2 activity and therefore a change in the balance of Th1 and Th2 normally observed in this infection model [101]. Additional studies using this model showed that THC injection suppressed Th1 immunity by inhibiting the production of IFN-γ and IL-12 as well as the expression of IL-12 receptors, and in addition, drug treatment increased the expression of the Th2-promoting cytokine, IL-4 [102]. It was also demonstrated, using CB1 and CB2 antagonists, that both receptors were involved in this THC-induced Th cell biasing. Other laboratories have reported Th biasing effects of cannabinoids (Fig. 5) . Splenocytes taken from THC-injected mice and stimulated in vitro with Con A were shown to produce reduced amounts of IL-2 and IFN-γ [103] and Propionibacterium acnes-primed mice injected with LPS showed a decreased production of IL-12 and increase of IL-10 when pretreated with HU210 and other cannabinoids [104]. Biasing toward Th2 immunity was also reported in a murine tumor model [105]. Mice injected with tumor cells and THC showed enhanced tumor growth, depressed cell-mediated immunity, and increased Th2 activity such as production of IL-10 and TGF-β, and these effects were attenuated by treatment with the CB2 antagonist, SR144528. A similar shift to Th2 cytokines was demonstrated in response to THC in activated peripheral blood T cell cultures [106]. In drug-treated cultures, proliferation was inhibited along with Th1 cytokines; Th2 cytokines were increased, and the CB2 antagonist inhibited the effects [106]. There is now ample evidence that cannabinoids and other GPCR agonists can modulate the development of Th (Fig. 5) . In the case of cannabinoids, it is possible that CB1 and CB2 may be differentially expressed on different subpopulations of APCs and Th cells. This selective expression could lead to an increase in Th2 development and a decrease in the development of Th1 cells, resulting in decreased cell-mediated immunity and increased antibody immunity. Continued research on the distribution and function of cannabinoid receptors as well as the production of endocannabinoids by different immune cells will provide greater insight into these mechanisms.

Cannabinoids and chemotaxis

Chemokine receptors, as noted above, are GPCR. It appears that other receptors of this group such as those for opioids and cannabinoids also are linked to cell-migration gene programs. For example, opioids are chemotactic when added to cultures of human blood monocytes and neutrophils, and these drugs can cross-desensitize cells to chemotaxis in combination with chemokines such as CCL5 [107]. These and other results provided evidence that the process of heterologous desensitization was occurring among chemotactic receptors that have varying affinities for different ligands such as chemokines and opioids. Cannabinoids also appear to have chemotactic activity for various cell types. HL60 cells expressing CB2 receptors were shown to migrate in response to CP55,940, and the migration could be demonstrated in experiments measuring chemotaxis and chemokinesis [108]. Drug treatment was also shown to increase the production of various cytokines and chemokines. Other cell types of immune lineage have been shown to migrate in response to cannabinoids. Myeloid leukemia cells expressing CB2 receptors displayed chemotaxis and chemokinesis in response to 2-AG but not in response to a variety of other cannabimimetic agents, and the migration was enhanced in the presence of IL-3 or granulocyte macrophage-colony stimulating factor [109]. 2-AG was also chemotactic for mouse spleen cells, and it was concluded that a major function for the CB2 receptor on these cells was the regulation of migration [109]. Mouse microglia cells also were shown to migrate in response to 2-AG through a CB2-mediated mechanism, and this effect was antagonized by the nonpsychotropic cannabinoids, cannabinol and cannabidiol [110]. Finally, in contrast to these immune cell studies, nonimmune cell migration is suppressed in response to cannabinoids. Human umbilical vein endothelial cells, when precultured with cannabinoids for 18 h, showed reduced chemotaxis in response to lysophosphatidic acid [111]. The CB2 antagonist inhibited this effect, and cannabinoid treatment was shown to suppress other functions involved in angiogenesis. From these studies, it appears that cannabinoids are similar to opioids in modulating cell migration and suggest that at least one function of the immunocannabinoid system is to regulate migration of immune cells.

Cannabinoids and tumor suppression

Marijuana smoking has been related to an increased incidence of head and neck cancer (see Cellular effects section); however, paradoxically, cannabinoids have been shown to suppress tumor development. Anandamide and other cannabinoids induce apoptosis in human PBMC, and this was suggested to account for the drug-induced suppression of lymphocyte proliferation [112]. These drugs were also shown to induce apoptosis and decrease Bcl-2 in macrophages and splenocytes, and this was suggested to contribute to increased processing and release of IL-1 [113]. Anti-tumor effects also have been linked to cannabinoid induction of apoptosis. Established gliomas in rats regressed in response to intra-tumor injection of THC and WIN55,212 [114]. Treatment of C6 glioma cells in culture with cannabinoids induced apoptosis by a pathway involving cannabinoid receptors and discrete signaling pathways involving ceramide and ERK activation. Similar experiments were performed using the CB2-selective agonist, JWH-133 [115]. Again, regression of established C6 tumors was observed following intra-tumor injection. In addition, a CB2 antagonist inhibited the effects, and cultures of C6 cells were induced to apoptosis in response to treatment with cannabinoids [115]. In other studies, proliferation of C6 glioma cells was suppressed by cannabinoids, an effect mediated by cannabinoid and vanilloid receptors [116]; furthermore, a variety of human and mouse malignant immune cell lines were induced to apoptosis by cannabinoid treatment, were suppressed in vivo by cannabinoids, and were inhibited through CB2-mediated mechanisms [117]. From these studies, it appears that cannabinoids can induce apoptosis and the inhibition of tumors through CB1 and CB2 mechanisms, suggesting their potential as anti-cancer drugs.

In addition to effects mediated by apoptosis, cannabinoids appear to suppress tumor cell growth by several other mechanisms. One of these involves the suppression of prolactin receptors. Endocannabinoids and synthetic cannabinoids were observed to inhibit the proliferation of human breast cancer cells [118]. The effect was CB1-dependent and resulted from the down-regulation of the long form of the prolactin receptor. Subsequent studies showed that endocannabinoids such as anandamide suppress the receptor by inhibiting adenylyl cyclase and MAPK and also can inhibit nerve growth factor receptor by CB1- but not CB2-mediated mechanisms [119 , 120]. From this, it appears that in some tumor suppression systems, both cannabinoid receptors are not involved, as in the case of apoptosis, suggesting cannabinoids affect tumor growth by mechanisms other than programmed cell death. Finally, cannabinoids have been reported to suppress tumor growth in several other ways, such as inducing the accumulation of lipid droplets in the tumor cells [121] and the suppression of angiogenesis [111], again suggesting that these drugs have the potential to affect tumor growth in a variety of ways. In contrast, the CB2 receptor has been reported to have oncogenic activity by inhibiting neutrophil differentiation through MEK/ERK and phosphatidylinositol-3 kinase signaling pathways [109]. It appears that the effect of marijuana and cannabinoids on tumor growth and development is exceedingly complex, showing enhancing and suppressive effects. This would suggest that the application of these agents to tumor therapy should proceed slowly and with consideration of all possible outcomes.

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SUMMARY

There is increasing effort to define and understand the mechanisms of the brain-immune connection. Many immune cytokines and brain neurochemicals engage in cross-talk between the immune and brain systems, orchestrating the regulation of immunity and chronic inflammatory diseases of the brain. The immunocannabinoid system of receptors and endogenous ligands appears to be a component of the brain-immune connection, and the details of its structure and function are now being elucidated. Immune cells express CB2 receptors and most probably, CB1 receptors, but considerable work is still needed to understand the distribution of these receptors among the various immune subpopulations, and in addition, a greater understanding is needed to define the factors that regulate receptor expression. It also is apparent that cannabinoid receptor signaling regulates the expression of various genes in immune cells; however, the scope and mechanisms of these gene activation programs await further definition. Cannabinoid receptors are GPCR, and the importance of this family of receptors in immune cell function is increasingly recognized. Regulatory functions are emerging for these receptors involving the mechanisms of Th cell biasing as well as chemotaxis of leukocytes and the regulation of apoptosis and tumor suppression. These developments involving the cannabinoid system and immune regulation suggest the possibility that the components of this system will be exploited in the future for therapy of chronic diseases and immune deficiencies. Furthermore, continued work in this area will lead to a better understanding of immune regulation in general and the brain-immune connection in particular.

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Acknowledgments

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From <http://www.jleukbio.org/content/74/4/486.full>

This article is sponsored by CannaVest, one of the leading suppliers of agricultural hemp-derived CBD from seed to finished products.

The biological mechanisms that control mood, anxiety, stress, fear, and other emotional behavior have been focused almost exclusively on the role of certain neurotransmitters called monoamines. While monoamines like dopamine, serotonin, and norepinephrine play an important role in controlling mood and anxiety, the vast majority of drug-based therapies that target those "monoaminergic" systems are limited in their efficacy.

More recently, research has begun to highlight other neurochemical systems, including cytokines, peptides, and bioactive lipids. By diving deeper into the science of bioactive lipids, we begin to unearth the potential role of the endocannabinoid system (ECS) in stress and fear responses.

The Endocannabinoid System's Role in the Human Body

The ECS has been described in our previous article as a complex physiologic network within the human body comprised of cannabinoid receptors (CB1 and CB2). There are endogenous cannabinoid compounds such as anandamide and 2-AG, and their respective enzymes responsible for maintaining balance in the system by regulating the synthesis and breakdown of the active endocannabinoid compounds.

Not surprisingly, cannabinoid receptors and the biochemical machinery necessary to synthesize and generate cannabinoids are present within areas of the brain known to control emotional behavior, mood, stress, and fear. These structures include the prefrontal cortex, amygdala, hippocampus, and periaqueductal gray (PAG) of the midbrain.

What Happens in the Brain When Cannabinoid Receptors Are Activated?

Preclinical studies in mice have clearly shown that activation of cannabinoid receptors within the PAG via direct injection of anandamide reduces panic and anxiety behavior, or the "fight or flight" phenomenon. Other studies using a similar model in animals have shown decreased fear-avoidant and conditioned responses to pain or punishment.

However, before we jump the gun and feel like we have fear, anxiety, and stress responses all figured out, it's not as simple as activating receptors through cannabinoid administration. Other animal studies utilizing systemic injections of Δ9-THC (not directly into the PAG of the midbrain) have demonstrated mixed responses, eliciting either anxiogenic (promoting increased levels of anxiety) or anxiolytic (decreasing levels of anxiety).

This bidirectional or paradoxical response appears to be dose-dependent, where lower doses seem to reduce stress, anxiety, and panic, while higher doses may promote increased feelings of stress, panic, and fear. These opposing effects are likely a result of the involvement of other neurotransmitter circuits within the brain that, when inhibited, actually create a hyper-excitable response of stress, anxiety, and fear.

Striking a Balance within the Cannabinoid System

To illustrate what neuroscientists currently feel is happening, think of being in a car with the brake pedal and accelerator. When the cannabinoid system is functioning to decrease fear, stress, and panic, the foot is being taken off of the accelerator in the areas of PAG-midbrain centers.

On the other hand, when dosing is too high, or there is an imbalance in the endocannabinoid system, whereby CB1 receptors are being over-activated in lieu of CB2, that's like taking the foot off the brake pedal, thereby removing inhibition that was normally in place and allowing the engine to rev higher—increased fear, anxiety, and stress from activation of other circuits in the brain that are normally applying the brake.

More recently, other preclinical research has demonstrated the important role of maintaining a healthy endocannabinoid system "tone" for improved stress resilience and reducing the residual post-traumatic anxiety, fear, and panic behaviors in mice that were chronically exposed to stress. Interestingly, the greater the exposure to stress, the greater the magnitude of the response to treatment (by improving the ECS and cannabinoid signaling).

Are Cannabinoid Compounds the "Gatekeepers" to Healthy Stress Levels?

One theory is that anadamide and 2-AG are acting as "gatekeepers" to keep the stress response and high emotional loads at bay and improving recovery to pre-stress levels once the stress burden is reduced to baseline. Moreover, there is evidence from preliminary human studies that disrupting endocannabinoid signaling and regulation has important impacts on the HPA (hypothalamic-pituitary-adrenal) axis by increasing signs of anxiety and depression.

There is an emerging role in the interplay between the immune system and the neuro-endocrine system, such that the field of psychoneuroimmunology has exploded with new basic and preclinical research over the past 5 years. The crosstalk between the immune system, cytokine signaling, and the nervous system influencing behavior, anxiety, and mood has been proposed by numerous preclinical studies. A number of recent studies have demonstrated a role of CB2 receptors classically associated with the immune system in anxiety and depression-related behavior in animal models and human clinical studies.

In this context, it's interesting to note that a balanced ECS appears to be pivotal to a healthy stress response and mitigating fear, anxiety, and panic that tend to accumulate with dysfunctional responses to stress.

CBD and Anxiety Reduction in Humans

A randomized, double-blind, placebo controlled trial in 15 humans demonstrated that up to 600 mg of CBD (cannabidiol) reduced measured anxiety compared to increased levels with a 10 mg dose of Δ9-THC. CBD appears to activate other receptors outside CB2, including 5HT1A and TRPV1, both of which are involved in the anxiolytic and mitigating panic/fear responses to stress.

The totality of the best available scientific evidence points to the importance of balancing the tone of the endocannabinoid system for supporting a healthy stress response. This preclinical and emerging human clinical data support the use of a CBD-rich or CBD-overweighted extract of cannabis for modulating fear, anxiety, and a healthy stress response.

Addressing Your CBD Questions

If you have questions about CBD, send them to social@leafly.com or share them via Facebook or Twitter. Our goal is to spread awareness of and education about hemp-derived CBD products, so send us your questions and we'll address them in future installments!

From <http://www.leafly.com/news/health/the-endocannabinoid-system-and-cbds-role-in-stress-anxiety-and-fe>

A Survey of American Medical School's Acceptance of the Science of the ECS (Endocannabinoid System)

October 14, 2014October 14, 2014 / HashManRiver

Introduction

The discovery of the endocannabinoid system (ECS) is the single most important scientific medical discovery since the recognition of sterile surgical technique. As our knowledge expands, we are coming to realize that the ECS is a master control system of virtually all physiology. The total effect of the ECS is to regulate homeostasis and prevent disease and aging. The more we learn, the more we realize that we are in the infancy of this scientific field of study. The ECS is a control system which involves tissue receptor proteins, cellular communication and control, molecular anatomy and the scavenging of oxygen free radicals. This new field of science will change medicine forever and prove cannabis the gold standard for many disease processes. Its effect on scavenging oxygen free radicals is applicable to all disease processes and this is why it has such wide medical application and is considered a cure-all by many.

The discovery of the ECS will replace the current medical system of managing and treating disease. Instead of management of symptoms after disease has occurred, we will prevent disease and cancer by manipulation of the ECS.

Research and education of medical students involving the ECS is being intentionally restricted by politics. No justification can be made for the restriction of the scientific study of cannabis and the endocannabinoid system. What is the danger of providing government-grown and tested cannabis to researchers? Diversion of research cannabis for non-scientific or recreational purposes does not seem to be a serious threat to national security.

Methods

The directors of the curriculum of the 157 accredited American medical schools (across all 50 states), were contacted initially by phone, then follow-up email. We asked the following questions:

1. Do you have a department of ECS (Endocannabinoid Science) with a director?
2. Do you teach the ECS as an organized course?
3. Do you mention the ECS in any ancillary classes like pathophysiology, neuroanatomy or pharmacology?

Resistance to the Survey

Our surveyors found a lot of resistance to answering questions, and we had to alter our technique for asking these pointed questions. Many people were unaware of what the word endocannabinoid actually meant. We were asking the medical schools if they were teaching a subject that many of them had no knowledge of. We initially called stating Dr. David Allen, a retired cardiothoracic and vascular surgeon, was sponsoring the study. This led to reluctance, suspicion and refusal to answer our questions. We had reactions like, "Who wants to know this?" and "Why do you want to know this information?" We quickly realized there was significant resistance to our questions. We then decided to pose as medical students looking to apply to an institution that specialized in the study of the ECS. This worked well, and we were able to acquire many responses from the office staff of curriculum directors. The directors were frequently not available and often too busy to talk to us. However, this change in technique made data collection easier and curriculum staff more cooperative and affable.

Here are some of the responses to the questions asked in this survey, ranging from passive-aggressive to blissfully ignorant:

- I doubt you will find a school that has a department of endocannabinoid science.
- How do you spell endocannabinoid?
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- Are you aware what type of institution we are? Why would we offer that here?
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- You will have some very brief exposure to cannabinoid receptors and their effects in our curriculum.
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However, a few responses gave our team some hope for the future of this science.

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- We do not teach the endocannabinoid system, but we do have a research lab dedicated to that issue.
- Three of our physicians include the endocannabinoid system in a lecture.
- VCU (Virginia Commonwealth University School of Medicine) has a leading researcher in the area of cannabinoids. There are numerous possibilities to attend seminars on the latest research in that area.

Some may criticize this study's accuracy, but the resulting numbers are so overwhelming that, even considering room for error, there is a clear failure of the medical establishment to overcome political repression of scientific knowledge. There is no reason our potential medical doctors should be ignorant of this important physiologic control mechanism. This is similar to ignoring a medical field like neuroanatomy.

The majority of people in the United States have no idea of the remarkable, scientifically-proven medical benefits of cannabis. These cannabinoids are responsible for massive reductions in diabetes, stroke and myocardial scars. Many cancers show significant responses to cannabinoids by

1. Inhibition of growth of the tumor
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Glucose and fatty metabolism, pain control and inflammation are all controlled by the ECS. There are many reports of patients with seizures that are unresponsive to all medicines except cannabis extracts. We are learning that even the sperm implantation into the ovum requires endocannabinoids for success. Mother's milk contains endocannabinoids that stimulate the infant to feed and thrive. (This transfer of cannabinoids from mother to child is the only still legal transfer of exogenous cannabinoids).

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Obviously, there can be no justification for repression of scientific study on any subject, especially the scientific study of a plant with unprecedented medical benefits and a lack of harmful side effects. When scientific knowledge is illegal, the truth does not exist. Americans for Safe Access (ASA) has a very illuminating study on the political repression of the scientific study of cannabis in this country that is well worth reading:
< http://americansforsafeaccess.org/downloads/Research_Obstruction_Report.pdf>

Results
The results of the study are predictable, so no one should be surprised! Not one of the medical schools surveyed had a department of endocannabinoid science or an ECS director. None of them taught the endocannabinoid science as an organized course. Only 21 of the 157 schools surveyed had the ECS mentioned in any course. 21/157 = 13.3%
In the United States of America, only 13% of the medical schools surveyed teach the endocannabinoid science to our future doctors.

Conclusion
The purpose of this study was to point out the absurdity of ignoring a new science. We would hope this study causes medical schools to rethink their position and welcome this new science. We call on the Deans of all medical schools to start organized courses in cannabinoid receptor science and its modulating effects on homeostasis. If the medical schools in your state do not teach the endocannabinoid science, you can discuss ways to incorperate the study of the ECS into their curriculum.
Humans have a hard time believing in things they can't see. Prior to the invention of the microscope, no medical schools taught sterile technique to their students because bacteria were unseen. You should research the name Ignaz Semmelweis and how the medical community treated his epiphany and scientific medical discovery. Dr Semmelweis referred to the physicians of the day as "ignoramuses." It may be time for today's physicians to catch up with the science of the times. The discovery of the ECS is proving it to be a master control system for physiology.
Recent studies show long-term use of cannabis decreases the incidence of diabetes by 66%. US patents prove CBD, a component of cannabis, decreases the size of a stroke by 50%. Other studies show a 66% reduction in the size of a heart scar after a heart attack. The sum of this information means that cannabis augments the body's own natural endocannabinoid system. Cannabis is not just a pharmaceutical; it is more properly termed a nutraceutical. Your body needs cannabis on a nutritional basis to prevent disease. Consumption of raw or uncooked cannabis, does not get you high, but has miraculous anti-oxidant effects from the acid forms of cannabinoids. You don't need a medical diagnosis to benefit from cannabis, as everyone can benefit from the antioxidant effects. As you can see, this concept will change medicine as we know it, including the distribution of money. The business of medicine will no longer be treatment-based, it will be prevention-based.
The big picture is that the ECS is a control system of physiology that will not be ignored. You can pretend the ECS doesn't exist or question its importance. People once questioned the significance of handwashing prior to surgical procedures because bacteria are unseen by the naked eye. People may not understand the complex science behind this medicine, but they know immediately when they use cannabis that it has some beneficial effects. The price that people are willing to pay for cannabis should be a good barometer on how well people think the medicine works. Only organized, double blinded scientific studies of cannabis will prove its true benefits or potential harm. Unfortunately, the politics behind prohibition prevent this from happening to any significant degree.
Ask yourself, "Why is scientific study of cannabis and the ECS restricted in the USA? What possible justification could explain this? Why should the education of medical students exclude this critical control system of physiology and health?" We encourage you to call the Dean of your local medical school and ask if they plan on teaching this science to our future doctors. When will this war end and the healing start? All wars mean big business for somebody. This war is being funded, and that is why it still continues. Stop the funding and the war will end.
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From <https://hashmanriver.wordpress.com/2014/10/14/a-survey-of-american-medical-schools-acceptance-of-the-science-of-the-ecs-endocannabinoid-system/>

By Nathan Stewart, Sioux Colombe, Ron Mullins, David Allen M.D.
Medical Cannabis Evaluations, Sacramento, CA

David B. Allen M.D.
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Glucose and fatty metabolism, pain control and inflammation are all controlled by the ECS. There are many reports of patients with seizures that are unresponsive to all medicines except cannabis extracts. We are learning that even the sperm implantation into the ovum requires endocannabinoids for success. Mother's milk contains endocannabinoids that stimulate the infant to feed and thrive. (This transfer of cannabinoids from mother to child is the only still legal transfer of exogenous cannabinoids). Prohibitionists will claim that no political repression of the science exists. They will claim that here are adequate scientific studies being funded, but the application process for scientific study of cannabis is nearly impossible to complete successfully. Dr. Donald Abrams is famous for being rejected for submitting numerous cannabis studies designed to prove benefits from this medication. After many denials, Dr. Abrams changed his tactic and asked to do a study to ascertain if cannabis caused changes in AIDS drugs that would make them less effective or more toxic. This study was approved for government funding because it asked to study the negative effects of cannabis.
Fortunately, this study showed cannabis does not make the AIDS drug more toxic or less effective. The Catch 22 for cannabis is that, in effect, the government states, "There are no studies that prove cannabis safety, therefore, it should remain in a class of dangerous drugs that are prohibited from scientific study." Because cannabis has not been studied, it is a Schedule I drug. Because it is a Schedule I drug, it cannot be studied. Obviously, there can be no justification for repression of scientific study on any subject, especially the scientific study of a plant with unprecedented medical benefits and a lack of harmful side effects. When scientific knowledge is illegal, the truth does not exist. Americans for Safe Access (ASA) has a very illuminating study on the political repression of the scientific study of cannabis in this country that is well worth reading:

When scientific knowledge is illegal, the truth does not exist. Americans for Safe Access (ASA) has a very illuminating study on the political repression of the scientific study of cannabis in this country that is well worth reading:

Ignorance Is Not Bliss!

Results
The results of the study are predictable, so no one should be surprised! Not one of the medical schools surveyed had a department of endocannabinoid science or an ECS director. None of them taught the endocannabinoid science as an organized course. Only 21 of the 157 schools surveyed had the ECS mentioned in any course. 21/157 = 13.3% In the United States of America, only 13% of the medical schools surveyed teach the endocannabinoid science to our future doctors.

Conclusion
The purpose of this study was to point out the absurdity of ignoring a new science. We would hope this study causes medical schools to rethink their position and welcome this new science. We call on the Deans of all medical schools to start organized courses in cannabinoid receptor science and its modulating effects on homeostasis. If the medical schools in your state do not teach the endocannabinoid science, you can discuss ways to incorperate the study of the ECS into their curriculum. Humans have a hard time believing in things they can't see. Prior to the invention of the microscope, no medical schools taught sterile technique to their students because bacteria were unseen. You should research the name Ignaz Semmelweis and how the medical community treated his epiphany and scientific medical discovery. Dr Semmelweis referred to the physicians of the day as "ignoramuses." It may be time for today's physicians to catch up with the science of the times. The discovery of the ECS is proving it to be a master control system for physiology. Recent studies show long-term use of cannabis decreases the incidence of diabetes by 66%. US patents prove CBD, a component of cannabis, decreases the size of a stroke by 50%. Other studies show a 66% reduction in the size of a heart scar after a heart attack. The sum of this information means that cannabis augments the body's own natural endocannabinoid system. Cannabis is not just a pharmaceutical; it is more properly termed a nutraceutical. Your body needs cannabis on a nutritional basis to prevent disease. You don't need a medical diagnosis to benefit from cannabis, as everyone can benefit from the antioxidant effects. As you can see, this concept will change medicine as we know it, including the distribution of money. The business of medicine will no longer be treatment-based, it will be prevention-based. The big picture is that the ECS is a control system of physiology that will not be ignored. You can pretend the ECS doesn't exist or question its importance. People once questioned the significance of handwashing prior to surgical procedures because bacteria are unseen by the naked eye. People may not understand the complex science behind this medicine, but they know immediately when they use cannabis that it has some beneficial effects. The price that people are willing to pay for cannabis should be a good barometer on how well people think the medicine works. Only organized, double blinded scientific studies of cannabis will prove its true benefits or potential harm. Unfortunately, the politics behind prohibition prevent this from happening to any significant degree. Ask yourself, "Why is scientific study of cannabis and the ECS restricted in the USA? What possible justification could explain this? Why should the education of medical students exclude this critical control system of physiology and health?" We encourage you to call the Dean of your local medical school and ask if they plan on teaching this science to our future doctors. When will this war end and the healing start? All wars mean big business for somebody. This war is being funded, and that is why it still continues. Stop the funding and the war will end. Jury nullification and education of the public on the truth about cannabis are the best ways to stop this war on us. Read and teach.

Resources:
US Patent; New use for Cannabinoids;
US Patent: Treating or preventing diabetes with Cannabidiol;
US Patent: Cannabinoids as antioxidants and Neuroprotectants;
US Patent: Phytocannabinoids in the treatment of cancer;
US Patent: Medical use for acidic cannabinoids;

From <http://blog.quantum9.net/ignorance-is-not-bliss-a-survey-of-american-medical-schools-acceptance-of-the-science-of-the-ecs-endocannabinoid-system/>

From <https://mail.google.com/mail/u/0/>

Breeding programme
GW's team includes experts in Cannabis breeding. In the genetic model used, the cannabinoid content of each chemical phenotype (chemotype) is controlled by four independent loci. By manipulating the genes at these four positions, our scientists can precisely control the cannabinoid composition of a plant. This is explained in the diagram below:

The gene at locus O allows the production of the initial phenolic precursors (resorcinolic acids). These combine with geranyl pyrophosphate to create the intermediate cannabinoids CBG and/or CBGV, the central precursors for the end-product cannabinoids THC(V), CBD(V) and CBC(V). The functional allele O is co-dominant; O/o hybrids have a low cannabinoid content and o/o plants are cannabinoid-free.

The ratio of propyl- and pentyl cannabinoid precursors is determined by a postulated locus A, which is still under investigation.

The CBG/CBGV intermediate is further processed by the alleles of locus B. BD and BT are co-dominant; the BD gene converts CBG(V) into CBD(V) and the BT gene converts CBG(V) into THC(V). In the BD/BT genotype, codominance allows the expression of a mixed CBD/THC chemotype. Also at this locus, non-functional alleles, designated B0 can exist; these are unable to convert the CBG(V) intermediate and leave the plant with a CBG(V) predominant chemotype.

Locus C is fixed so all plants have CBC synthase activity. CBC synthase competes for the same CBG(V) precursor as the synthases encoded by locus B (THC and/or CBD synthase). In 'normal' Cannabis plants, CBC synthase is only active in the juvenile state. However, our scientists have discovered genetic factors that induce morphological mutations that are associated with a 'prolonged juvenile chemotype'. Prototype CBC production plants carry these factors in combination with B0/B0 at locus B. In these plants CBC synthase has no competition from THC or CBD synthase.

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Meijer EPM de, Hammond KM. 2005. The inheritance of chemical phenotype in Cannabis sativa L. (II): cannabigerol predominant plants. Euphytica145: 189-198.

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Meijer EPM de, Hammond KM, Sutton A. 2009. The inheritance of chemical phenotype in Cannabis sativa L. (IV): cannabinoid-free plants. Euphytica 168: 95-112.

Licensed and controlled cultivation
GW is licensed by the UK Home Office. Cultivation of GW's first Cannabis plants began in August 1998. Selected seedlings are maintained as clones. Clones are genetically identical, thus ensuring that the ratio of plant constituents is fixed within narrow limits. Clonal propagation does not involve genetic modification.

GW's botanical research team continues to develop new chemotypes. These will produce the raw material bases for future pharmaceutical research.

GW's Cannabis plants are grown in highly secure computer-controlled glasshouses. All aspects of the growing climate, including temperature, air change and photoperiod, are computer-controlled and the plants are grown without the use of pesticides. Careful control of the growing environment ensures that GW’s plant material is grown to very strict pharmaceutical standards and that growth is phased to ensure continuity of supply.

Cultivation capability has been increased to cater both for commercial supply of our first product Sativex® and research quantities of novel chemotypes for the production of other medicines. Pharmaceutical production capacity has also been scaled up, both in-house and through external contractors, to supply tens of thousands of patients.

High levels of chemical consistency are important in applications made to medical regulatory authorities. Routine laboratory analysis demonstrates that GW’s botanical raw materials meet strict specifications of quality.

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From <http://www.gwpharm.com/cultivation.aspx>

The Cannabinoid Receptor System

Only in the last two decades, a natural cannabinoid receptor system has been discovered in the human body. It is by interacting with these receptors that cannabinoids exert many of their pharmacological effects. The discovery of the cannabinoid receptor system has sparked renewed interest in the therapeutic potential of cannabinoids by providing important new targets for drugs. There are at least two types of cannabinoid receptors in mammalian tissues, CB1 and CB2. CB1 receptors are present in the brain and spinal cord and in certain peripheral tissues. CB2 receptors are expressed primarily in immune tissues. There is preliminary evidence to suggest that additional cannabinoid receptor types may exist.

Receptor Distribution

CB1 receptors are widely distributed but are particularly abundant in some areas of the brain including those concerned with movement and postural control, pain and sensory perception, memory, cognition, emotion, autonomic and endocrine functions. They are also found in appetite regulating areas such as the hypothalamus as well as reward centres such as the lymbic system and have therefore been implicated in food intake. More recently, CB1 has been isolated in tissues that are important for energy metabolism such as the liver, adipose (fat) tissue and skeletal muscle. The second type of receptor, the CB2 receptor, can mediate regulation of cytokine release from immune cells and of immune cell migration in a manner that seems to reduce inflammation and certain kinds of pain.

So although the endocannabinoid system interacts with many neurotransmitter/neuromodulator systems it is important to note that phytocannabinoids have the ability to interact with all sorts of cellular pathways implicated in a range of diseases such as cancer and metabolic syndrome.

Receptor Modulation

Cannabinoids act as ligands (a small molecule able to dock onto the binding site of a protein) conferring their ability to modulate a receptor's behaviour and consequently their downstream biological pathways. Although the phytocannabinoids all have similar structures, they display a remarkably wide array of actions at each of the different receptors that are now thought to contribute to the endocannabinoid system (such as cannabinoid receptors, transient receptor potential [TRP] channels, melatonin and serotonin receptors, the PPARs and a host of orphan G-coupled receptors). For example it is known that THC positively regulates the CB1 receptor whereas it is negatively regulated by THCV; interestingly CBD has very little action at this site whatsoever.

It is important to note that terms positive and negative regulation can be broken down further into a range of subtle but important physiological actions.

Over the last few decades, when the pharmaceutical industry discover a receptor responsible for a particular disorder, they screen batteries of small molecules and compounds to identify any that could be used to treat that disorder. Often they have concentrated on those that exert the greatest effects, often referred to as full agonists or inverse agonists, in the hope that very small doses could lead to improved symptoms within patients in the clinic. Clearly there are circumstances where highly active compounds leading to absolute maximum or minimum receptor regulation may not offer the optimum pharmaceutical profile. In some families of receptors, especially those that have a constitutive activity, a partial agonist would offer a better solution. Endocannabinoids act as partial agonists, playing modulatory roles, and because phytocannabinoids behave in a similar fashion they can offer help within a dysregulated endocannabinoid system. GW are particularly well placed to explore these therapeutic advantages.

Positive Regulation:

This animation differentiates full agonists (usually synthetic compounds) and partial agonists (especially useful in systems such as the ECS which are under tonic control):

Negative Regulation:

This animation illustrates the difference between inverse agonism and neutral antagonism at a constitutively active receptor:

Regulation of Neurotransmission

As explained above, neurotransmitter modulation covers just one aspect of the mechanisms that govern cannabinoids therapeutic abilities. However as it was the focus of much early research within the field it is by far the most fully understood mechanism of action.

[1]Information is transmitted around the body in the form of electrical impulses that travel through nerves. As the signal reaches the end of the nerve, or axon, the resulting depolarisation stimulates the release of stored vesicles of neurotransmitters (the yellow molecules). These traverse the synapse (the gap dividing two nerves) where they bind to receptors on the post-synaptic cell. Activation of these post-synaptic receptors then initiates a series of events.

[2]One of these series of events is the release of Endocannabinoids (the red molecules) which are synthesised and released locally and function as a retrograde transmitter.

[3]The endocannabinoids travel in the opposite direction to the initial neurotransmitters, backwards across the synaptic cleft, and bind to pre-synaptic CB1 receptors (the light blue receptors). This feedback allows pre-synaptic regulation of transmitter release whereby the binding of endocannabinoids

will retrogradely inhibit the release of further neurotransmitters, whether the neurotransmitters are inhibitory (e.g. GABA) or excitatory (e.g. glutamate)
[4]Phytocannabinoids are able to mimic the action of these endocannabinoids. In this way, they are able to augment the effect that endocannabinoids have in regulating the transmission of impulses from one nerve to another.

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From <http://www.gwpharm.com/mechanism-of-action.aspx>

Cannabis, cannabinoids and cancer – the evidence so far
Category: Science blog July 25, 2012 Kat Arney

Cannabis has long been used for medicinal as well as recreational purposes. Image source
[Please note – we are updating this blog post regularly as new research is published.]
Few topics spark as much debate on this blog and on our Facebook page than cannabis.
So we thought we'd take a look at the common questions raised about the evidence and research into cannabis, cannabinoids (the active chemicals found in the plant and elsewhere) and cancer, and address some of the wider issues that crop up in this debate.
We've broken it down under a number of headings:
• What are cannabinoids and how do they work?
• Can cannabinoids treat cancer? (including lab research, clinical research and unanswered questions)
• Can cannabis prevent or cause cancer?
• What about controlling cancer symptoms such as pain or sickness?
• Is Cancer Research UK investigating cannabinoids?
• It's natural so it must be better, right?
• "Have you seen this video? This guy says cannabis cures cancer!"
• "It's all a big conspiracy – you don't want people to be cured!"
• "What's the harm? There's nothing to lose."
• "Big Pharma can't patent it so they're not interested."
• "Why don't you campaign for cannabis to be legalised?"
This post is long, but can be summarised by saying that at the moment there isn't enough reliable evidence to prove that cannabinoids – whether natural or synthetic – can effectively treat cancer in patients, although research is ongoing around the world.
Read on to get the full picture.
What are cannabinoids and how do they work?
"Cannabinoids" is a blanket term covering a family of complex chemicals (both natural and man-made) that lock on to cannabinoid receptors – protein molecules on the surface of cells.
Humans have been using cannabis plants for medicinal and recreational purposes for thousands of years, but cannabinoids themselves were first purified from cannabis plants in the 1940s. The structure of the main active ingredient of cannabis plants – delta-9 tetrahydrocannabinol (THC) – was discovered in the 60s. It wasn't until the late 1980s that researchers found the first cannabinoid receptor, followed shortly by the discovery that we create cannabinoid-like chemicals within our own bodies, known as endocannabinoids.

The CB1 and CB2 receptors. Image source
We have two main types of cannabinoid receptor, CB1 and CB2, which are found in different locations and do different things. CB1 is mostly found

on cells in the nervous system, including certain areas of the brain and the ends of nerves throughout the body, while CB2 receptors are mostly found in cells from the immune system. Because of their location in the brain, it's thought that CB1 receptors are responsible for the infamous 'high' (known as psychoactive effects) resulting from using cannabis.

There is also a third family of cannabinoid receptors, known as GPR, of which the main type is GPR55. Less is known about these receptors, but researchers are investigating them to see if they could lead to effective approaches for treating cancer. [Updated KA 14/08/14]

Over the past couple of decades scientists have found that endocannabinoids and cannabinoid receptors are involved in a vast array of functions in our bodies, including helping to control brain and nerve activity (including memory and pain), energy metabolism, heart function, the immune system and even reproduction. Because of this molecular multitasking, they're implicated in a huge range of illnesses, from cancer to neurodegenerative diseases.

Can cannabinoids treat cancer?

There is no doubt that cannabinoids – both natural and synthetic – are interesting biological molecules. Hundreds of scientists around the world are investigating their potential in cancer and other diseases – as well as the harms they can cause – brought together under the blanket organisation The International Cannabinoid Research Society.

Researchers first looked at the anticancer properties of cannabinoids back in the 1970s, and many hundreds of scientific papers looking at cannabinoids and cancer have been published since then. This Wellcome Witness seminar is also fascinating reading for aficionados of the history of medical cannabis, including the scientific, political and legal twists. [Updated KA 26/03/14]

But claims that this body of preclinical research is solid "proof" that cannabis or cannabinoids can cure cancer is highly misleading to patients and their families, and builds a false picture of the state of progress in this area. For example, we've taken a look at more than 30 scientific papers that are often claimed to "prove" that cannabis cures various types of cancer. [Updated KA 21/07/14]

Let's take a closer look at the evidence.

Lab research

Virtually all the scientific research investigating whether cannabinoids can treat cancer has been done using cancer cells grown in the lab or animal models. It's important to be cautious when extrapolating these results up to real live patients, who tend to be a lot more complex than a Petri dish or a mouse.

Virtually all the research into cannabinoids and cancer so far has been done in the lab.

Through many detailed experiments, handily summarised in this recent article in the journal Nature Reviews Cancer, scientists have discovered that various cannabinoids (both natural and synthetic) have a wide range of effects in the lab, including:

• Triggering cell death, through a mechanism called apoptosis
• Stopping cells from dividing
• Preventing new blood vessels from growing into tumours
• Reducing the chances of cancer cells spreading through the body, by stopping cells from moving or invading neighbouring tissue
• Speeding up the cell's internal 'waste disposal machine' – a process known as autophagy – which can lead to cell death

All these effects are thought to be caused by cannabinoids locking onto the CB1 and CB2 cannabinoid receptors. It also looks like cannabinoids can exert effects on cancer cells that don't involve cannabinoid receptors, although it isn't yet clear exactly what's going on there.

So far, the best results in the lab or animal models have come from using a combination of highly purified THC and cannabidiol (CBD), a cannabinoid found in cannabis plants that counteracts the psychoactive effects of THC. But researchers have also found positive results using synthetic cannabinoids, such as a molecule called JWH-133.

It's not all good news though, as there's also evidence that cannabinoids may also have undesirable effects on cancer.

For example, some researchers have found that although high doses of THC can kill cancer cells, they also harm crucial blood vessel cells, although this may help their anti-cancer effect by preventing blood vessels growing into a tumour. And under some circumstances, cannabinoids can actually encourage cancer cells to grow, or have different effects depending on the dosage and levels of cannabinoid receptors present on the cancer cells. [Edited for clarity and to add reference – KA 27/07/12]

Others have discovered that activating CB2 receptors may actually interfere with the ability of the immune system to recognise and destroy tumour cells, although some scientists have found that certain synthetic cannabinoids may enhance immune defences against cancer.

Furthermore, cancer cells can develop resistance to cannabinoids and start growing again, although this can be got round by blocking a certain molecular pathway in the cells known as ALK.

combining cannabinoids with other chemotherapy drugs may be a much more effective approach

And yet more research suggests that combining cannabinoids with other chemotherapy drugs may be a much more effective approach. This idea is supported by lab experiments combining cannabinoids with other drugs including gemcitabine and temozolomide.

As might be expected, whenever research about cannabis or cannabinoids hits the news there is a lot of interest on social media. But often it turns out that the hype don't realistically reflect the work. For example, this study from researchers at the University of East Anglia was done using cancer cells grown in the lab or transplanted into mice, to try and understand why different levels of purified THC seem to have different effects on cancer cells – something that has been noticed from previous experiments on cannabinoids and cancer cells.

The researchers found that THC seems to work through two different receptor molecules coming together – CB2 and GPR55 – and that high doses slow cancer cells growth while low doses don't. So they think that designing drugs that make sure the receptors come together in the right way to kill cancer cells could be a good way to harness the potential power of cannabinoids to treat cancer in a much more effective and targeted way.

But while it's an interesting scientific paper and helps to shed light on the molecular "nuts and bolts" that underpin how some cancer cells may respond to cannabinoids, and could point to ways to make cannabinoid drugs more effective in the future, it certainly doesn't tell us that cannabis can effectively treat cancer in patients at the moment.

Clinical research

But that's the lab – what about clinical research involving people with cancer? Results have been published from only one clinical trial testing whether cannabinoids can treat cancer in patients, led by Dr Manuel Guzman and his team in Spain. Nine people with advanced, terminal glioblastoma multiforme – an aggressive brain tumour – were given highly purified THC through a tube directly into their brain.

Eight people's cancers showed some kind of response to the treatment, and one didn't respond at all. All the patients died within a year, as might be expected for people with cancer this advanced.

The results from this study show that THC given in this way is safe and doesn't seem to cause significant side effects. But because this was an early stage trial, without a control group, it's impossible to say whether THC helped to extend their lives. And while it's certainly not a cure, the trial results suggest that cannabinoids are worth pursuing in clinical trials.

There is also a published case report of a 14-year old girl from Canada who was treated with cannabis extracts (also referred to as "hemp oil"), but there is limited information that can be obtained from a single case treated with a varied mixture of cannabinoids. More published examples with detailed data are needed in order to draw a fuller picture of what's going on. [Updated 26/03/14, KA]

Unverified anecdotes about 'cures' do little to help progress towards more effective treatments for patients on a wider scale – even if they do get published in newspapers, they aren't strong scientific evidence. In order to build a solid evidence base that might support future applications for funding or clinical trials it's important to gather together detailed information about individual cases.

Here in the UK, Dr Wai Liu at St George's University is researching cannabis and cannabinoids for treating cancer (we are not currently funding his work). You can read about some of his recent work here. He is happy to collect individual stories from UK patients, and can be contacted by email. In the US, the Office of Cancer Complementary and Alternative Medicine gathers similar stories for their Best Case Series. [Updated KA 21/07/14]

A handful of other clinical trials of cannabinoids are currently being set up. We are helping to support the only two UK trials of cannabinoids for treating cancer, through our Experimental Cancer Medicine Centre (ECMC) Network funded by Cancer Research UK and the devolved Departments

of Health. One early-stage trial is testing a synthetic cannabinoid called dexanabinol in patients with advanced cancer, and the other is an early-stage trial testing a cannabis extract called Sativex for treating people with glioblastoma multiforme brain tumours. [Edited to add more information about the trials – KA 22/08/12, KA 24/03/14]

Unanswered questions

There are still a lot of unanswered questions around the potential for using cannabinoids to treat cancer.

An antique bottle of cannabis extract. Image source

The biggest issue is that there isn't enough evidence to show that they can treat cancer in people, although research is still ongoing around the world. And it's not clear which type of cannabinoid – either natural or synthetic – might be most effective, what kind of doses might be needed, or which types of cancer might respond best to them. So far there have been intriguing results from lab experiments with prostate, breast, lung cancer, skin, bone and pancreatic cancers, glioma brain tumours and lymphoma. But the take-home message is that different cannabinoids seem to have different effects on various cancer types, so they are far from being a 'universal' treatment.

Most research has been focused on THC, which occurs naturally in cannabis plants, but researchers have found that different cannabinoids seem to work better or worse different types of cancer cells. Lab experiments have shown promising results with THC on brain tumour and prostate cancer cells, while CBD seems to work well on breast cancer cells.

Then there's the problem of the psychoactive effects of THC, particularly at high doses, although this can be counteracted by giving it together with CBD. Because of this problem, synthetic cannabinoids that don't have these effects might be more useful in the long term.

There are also big questions around the best way to actually get the drugs into tumours. Because of their chemical makeup, cannabinoids don't dissolve easily in water and don't travel very far in our tissues. This makes it hard to get them deep into a tumour, or even just deliver them into the bloodstream in consistently high enough doses to have an effect.

The clinical trial led by Dr Guzman in Spain involved directly injecting cannabinoids into patients' brains through a small tube. This isn't an ideal method as it's very invasive and carries a risk of infection, so researchers are investigating other delivery methods such as tablets, oil injections, mouth sprays or even microspheres.

We also don't know whether cannabinoids will help to boost or counteract the effects of chemotherapy, nor which combinations of drugs might be good to try. And there are currently no biological markers to help doctors identify who might benefit from cannabinoids and who might not – remember that one patient on the brain tumour trial failed to respond to THC at all.

None of these issues are deal-breakers, but these questions need answering if there's any hope of using cannabinoids to effectively and safely treat cancer patients.

there are hundreds of exciting potential cancer drugs being developed and tested in university, charity and industry labs all over the world – cannabinoids are merely a small part of a much larger picture

It's worth remembering that there are hundreds of exciting potential cancer drugs being developed and tested in university, charity and industry labs all over the world – cannabinoids are merely a small part of a much larger picture.

Most of these compounds will never make it into the clinic to treat patients for a huge range of reasons including toxicity, lack of effectiveness, unacceptable side effects, or difficulty of delivering the drug to tumours.

Without doing rigorous scientific research, we will never sift the 'hits' from the 'misses'. If cannabinoids are ever to get into clinical use, they need to overcome these hurdles and prove they have benefits over existing cancer treatments.

Can cannabis prevent or cause cancer?

So that's a brief look at cannabinoids to treat cancer. But can they stop the disease from developing? Or could they play a role in causing cancer?

There's controversy around the health risks of cannabis. Image source

In experiments with mice, animals given very high doses of purified THC seemed to have a lower risk of developing cancer, and there has been some research suggesting that endocannabinoids (cannabinoids produced by the body) can suppress tumour growth. But there's no solid scientific evidence at the moment to show that cannabinoids or cannabis can cut the risk of cancer in people.

When it comes to finding out whether cannabis can cause cancer, the evidence is a lot murkier. This is mainly because most people who use cannabis smoke it mixed with tobacco, a substance that definitely does cause cancer.

This complex issue recently hit the headlines when the British Lung Foundation released a study suggesting that the cancer risks of cannabis had been underestimated, although this has been questioned by some experts including Professor David Nutt.

What about controlling cancer symptoms such as pain or sickness?

Although there's a lack of data showing that cannabinoids can effectively treat cancer, there is good evidence that these molecules may be beneficial in other ways.

As far back as the 1980s, cannabinoid-based drugs – including dronabinol (synthetic THC) and nabilone – were used to help reduce nausea and vomiting caused by chemotherapy. But there are now safer and more effective alternatives and cannabinoids tend to only be used where other approaches fail.

In some parts of the world – including the Netherlands – medical use of marijuana has been legalised for palliative use (relieving pain and symptoms), including cancer pain. For example, Dutch patients can obtain standardised, medicinal-grade cannabis from their doctor, and medicinal cannabis is available in many states in the US.

But one of the problems of using herbal cannabis is about dosage – smoking it or taking it in the form of tea often provides a variable dose, which may make it difficult for patients to monitor their intake. So researchers are turning to alternative dosing methods, such as mouth sprays, which deliver a reliable and regulated dose.

Large-scale clinical trials are currently running in the UK testing whether a mouth spray called Sativex (nabiximols) – a highly purified pharmaceutical-grade extract of cannabis containing THC and CDB – can help to control severe cancer pain that doesn't respond to other drugs.

There may also be potential for the use of cannabinoids in combating the loss of appetite and wasting experienced by some people with cancer, although a clinical trial comparing appetite in groups of cancer patients given cannabis extract, THC and a placebo didn't find a difference between the treatments.

Is Cancer Research UK investigating cannabinoids?

We want to see safe, reliable and effective treatments become available for patients as quickly as possible. We receive no government funding for our research, and it is all paid for by the generosity of the public. This is obviously not a bottomless purse, and we do not have financial reserves to draw on.

Because of this limitation, we can only fund the very best research proposals that come to us that will bring benefits to people with cancer. We've previously written in detail about how we fund research projects.

Cancer Research UK has funded research into cannabinoids, notably the work of Professor Chris Paraskeva in Bristol investigating the properties of cannabinoids as part of his research into the prevention and treatment of bowel cancer. He has published a number of papers detailing lab experiments looking at endocannabinoids as well as THC, and written an interesting review looking at the potential of cannabinoids for treating bowel cancer.

Our funding committees have previously received other applications from researchers who want to investigate cannabinoids that have failed to reach our high standards for funding. If we receive future proposals that do meet these stringent requirements, then there is no reason why they would not be funded – assuming we have the money available to do so.

We support the only two UK clinical trials of cannabinoids for treating cancer through our national network of Experimental Cancer Medicine Centres, funded by Cancer Research UK and the devolved Departments of Health. One is an early-stage trial testing a synthetic cannabinoid called dexanabinol for people with advanced cancer, the other is an early-stage trial testing a drug called Sativex (an extract from cannabis plants) for people

with glioblastoma multiforme brain tumours. [Added 22/08/12 – KA, Updated KA 25/03/14]

"It's natural so it must be better, right?"

There's no doubt that the natural world is a treasure trove of biologically useful compounds. But whole plants or other organisms are a complex mix of hundreds of chemicals (not all of which may be beneficial) and contains low or variable levels of active ingredients. This makes it difficult to give accurate doses and runs the risk of toxic side effects.

Foxgloves – a source of medically useful chemicals. Image source

For example, foxgloves (Digitalis) are a useful source of chemicals called cardiac glycosides, first purified in 1785 – a date widely considered to be the beginning of modern drug-based medicine. These drugs are now used to treat many thousands of people around the world with heart failure and other cardiac problems. But the entire plant itself is highly toxic, and eating just a small amount can kill.

As another example, although the antibiotic penicillin was first discovered in a fungus, it doesn't mean that someone should munch some mould when suffering an infection. In fact, the bug-beating powers of 'natural' penicillin are confined to a relatively small range of bacteria, and chemists have subsequently developed a wider range of life-saving antibiotics based on the drug's structure.

Aspirin is another old drug, first discovered in the form of salicylic acid in white willow bark. But this naturally-occurring chemical causes severe stomach irritation, which led to the German company Bayer developing an alternative version – acetylsalicylic acid – which was kinder to the tummy. Aspirin is now arguably one of the most successful drugs of all time, and is still being investigated for its potential in preventing or even treating cancer.

Numerous potent cancer drugs have also been developed in this way – purifying a natural compound then improving it and testing it to create a beneficial drug – including taxol (originally from yew leaves); vincristine and vinblastine (from rosy periwinkles); camptothecin (from the Chinese Xi Shu tree); colchicine (from crocuses); and etoposide (from the May Apple). And we recently wrote about a clinical trial being run by our scientists to test whether curcumin, a purified chemical from the curry spice turmeric, could help treat people with advanced bowel cancer.

But it bears repeating that the fact that these purified drugs in controlled, high doses can treat cancer doesn't mean that the original plant (or a simple extract) will have the same effect. So although cannabis contains certain cannabinoids, it doesn't automatically follow that cannabis itself can treat cancer.

As we said above, there is no good evidence that natural cannabinoids, at the doses present in simple cannabis preparations, can treat cancer in patients. It's also completely unknown whether there may be any other chemicals in 'street' cannabis extracts that could be harmful to patients or even encourage tumour growth.

"Have you seen this video? This guy says cannabis cures cancer!"

There is a strong and persistent presence on the internet arguing that cannabis can cure cancer. For example, there are numerous videos and unverified anecdotes claiming that people have been completely cured of cancer with cannabis, hemp/cannabis oil or other cannabis derivatives.

YouTube videos are not scientific evidence.

Despite what the supporters of these sources may claim, videos and stories are not scientific evidence for the effectiveness of any cancer treatment. Extraordinary claims require extraordinary evidence – YouTube videos are emphatically not scientific evidence, and we are not convinced by them.

Based on the arguments presented on these kinds of websites, it's impossible to tell whether these patients have been 'cured' by cannabis or not. We know nothing about their medical diagnosis, stage of disease or outlook. We don't know what other cancer treatments they had. We don't know about the chemical composition of the treatment they got. And we only hear about the success stories – what about the people who have tried cannabis and not been cured? People who make these bold claims for cannabis only pick their best cases, without presenting the full picture.

This highlights the importance of publishing data from scientifically rigorous lab research and clinical trials. Firstly because conducting proper clinical studies enables researchers to prove that a prospective cancer treatment is safe and effective. And secondly because publishing this data allows doctors around the world to judge for themselves and use it for the benefit of their patients.

This is the standard to which all cancer treatments are held, and it's one that cannabinoids should be held to too. Internet anecdotes and videos prove nothing and benefit no-one – we need reliable, scientific research, which (as discussed above) is exactly what is going on.

"It's all a big conspiracy – you don't want people to be cured!"

As we've previously said, accusations that we are somehow part of a global conspiracy to suppress cancer cures are as absurd as they are offensive. Not only to the thousands of our scientists, doctors and nurses who are working as hard as they can to find more effective treatments for the complex set of challenging diseases we call cancer, but also the hundreds of thousands of people in the UK and beyond who support this life-saving work through generous donations of money, energy and time.

Our aim is to beat cancer through research

Our aim is to beat cancer, and we believe that the best way to do this through rigorous scientific research aimed at understanding cancer on a biological level and working out how to prevent, detect and treat it more effectively. This approach has helped to change the face of cancer prevention, diagnosis, treatment, leading to a doubling in survival rates over the past 40 years.

As a research-based organisation, we want to see reliable scientific evidence to support claims made about any cancer treatment, be it conventional or alternative. The claims made for many alternative cancer therapies still require solid evidence to support them, and it often turns out that these 'miracle cures' simply don't work when they're put to the test.

This doesn't mean there's a conspiracy to suppress the "True Cure for Cancer" – it means that doctors and researchers want to see solid evidence that the claims made by people peddling these treatments are true.

This is vital because lives are at stake. Some people may think that a cancer patient has nothing to lose by trying an alternative treatment, but there are big risks.

"What's the harm? There's nothing to lose."

If someone chooses to reject conventional cancer treatment in favour of unproven alternatives, including cannabis, they may miss out on treatment that could save or significantly lengthen their life. They may also miss out on effective symptom relief to control their pain and suffering, or the chance to spend precious time with their loved ones.

Furthermore, many of these unproven therapies come at a high price, and are not covered by the NHS or medical insurance. And, in the worst cases, an alternative therapy may even hasten death. Cannabis is also an illegal (class B) drug in the UK.

Although centuries of human experimentation tells us that naturally-occurring cannabinoids are broadly safe, they are not without risks. They can increase the heart rate, which may cause problems for patients with pre-existing or undiagnosed heart conditions. They can also interact with other drugs in the body, including antidepressants and antihistamines. And they may also affect how the body processes certain chemotherapy drugs, which could cause serious side effects.

There is also a reported case where a Dutch lung cancer patient took cannabis extract that had been bought from a street source. Within a matter of hours she was in hospital in a coma. This highlights the risks of taking 'street' cannabis extracts of unknown concentration and quality in an uncontrolled way, and accentuates the need for careful research into how best to use cannabinoids for treating patients.

Furthermore, there are other risks associated with using black market or home-made preparations of cannabis, particularly cannabis oil. For example, there may be toxic chemicals left from the solvents used in the preparation process. There is also a risk that pesticides found in cannabis crops may be concentrated in these preparations – recent research from California suggests high rates of such contaminants in samples from a range of different sources.

Finally, we are also aware of internet scams by people offering to sell cannabis preparations. As well as the risk of getting something with completely unknown chemical or medicinal properties and unknown effectiveness, there is a strong chance of receiving nothing at all. [Updated KA 14/08/14]

when conventional treatment fails, there is little chance that turning to an unproven alternative touted on the internet will provide a cure
It is a sad fact that although huge progress has been made over recent years, many thousands of people in the UK lose their lives to cancer every year – a situation that we urgently want to change through research. But when conventional treatment fails, there is little chance that turning to an unproven alternative touted on the internet will provide a cure.
In this situation, we recommend that cancer patients talk to their doctor about clinical trials that they may be able to join, giving them access to new drugs and providing valuable data that will help other sufferers in future.
"Big Pharma can't patent it so they're not interested."
Some people argue that the potential of cannabinoids is being ignored by pharmaceutical companies, because they can't patent the chemicals occurring in cannabis plants. But pharma companies are not stupid, and they are quick to jump on promising avenues for effective therapies.
As we've shown, there are hundreds of researchers around the world investigating cannabinoids, in both private and public institutions. And there are many ways that these compounds can be patented – for example, by developing more effective synthetic compounds or better ways to deliver them.
On the flip side, other people argue that patients should be treated with 'street' or homegrown cannabis preparations, and that the research being done by companies and other organisations is solely to make money and prevent patients accessing "The Cure". This is also a false and misleading argument, analogous to suggesting that patients in pain should buy heroin or grow opium poppies rather than being prescribed morphine by a doctor.
The best way to ensure that the benefits of cannabinoids – whether natural or synthetic – are brought to patients is through proper research using quality-controlled, safe, legal, pharmaceutical grade preparations containing known dosages of the drugs.
To do this requires time, effort and money, which may come from companies or independent organisations such as charities or governments. And, ultimately, this investment needs to be paid back by sales of a safe, effective new drug.
We are well aware of the issues around drug pricing and availability – for example, the recent situations with abiraterone and vemurafenib – and we are pushing for companies to make new treatments available at a fair price. We would also hope that if any cannabinoids are shown to be safe and effective enough to make it to the clinic, they would be available at a fair price for all patients that might benefit from them.
"Why don't you campaign for cannabis to be legalised?"
As things currently stand, cannabis is classified as a class B drug in the UK, meaning that it is illegal to possess or supply it.
It is not for Cancer Research UK to comment on the legal status of cannabis, its use or abuse as a recreational drug, or its medical use in any other diseases. But we are supportive of properly conducted scientific research that could benefit cancer patients.
In summary
At the moment, there simply isn't enough evidence to prove that cannabinoids – whether natural or synthetic – works to treat cancer in patients, although research is ongoing. And there's certainly no evidence that 'street' cannabis can treat cancer.
As a research-based organisation, we continue to watch the progress of scientists around the world for advances that may benefit people with cancer.
As a research-based organisation, we continue to watch the progress of scientists around the world for advances that may benefit people with cancer.
And although cannabinoid research is an interesting avenue, it's certainly not the only one.
Kat
Note: We've already entered into two lengthy, time-consuming and ultimately circular debates about cannabis, cannabinoids and cancer which you can read here and here.
Because of this, we are taking the unusual step of keeping public comments closed on this post, as we feel that we have fully laid out our position. If you have a considered comment you would like us to publish on this post you can contact the blog team at scienceblog.cancer.org.uk
Finally, we are grateful to Dr Manuel Guzman (Complutense University, Madrid), Professor Vincenzo di Marzo (Institute of Biomolecular Chemistry, Naples, and GW Pharmaceuticals) and Dr Wai Liu (St George's Hospital, London) for helpful discussions as we were writing this post.

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An important message for cancer patients: Beware the 'cannabis oil' scammers
We have become aware that scammers are tricking cancer patients and their families into handing over money for 'cannabis oil', yet receiving nothing in return. At least one of these fraudsters is using the email address ukcancerresearchcentre @ gmail.com and claiming to be based at 407 St John Street, London, which is the address…
April 20, 2015
In "Alternative/complementary"

Cannabinoids for treating cancer?
Read our in-depth post on cannabis and cannabinoids here Researchers in Spain have published results in this week's British Journal of Cancer showing that certain cannabinoids – molecules so-called because they were originally found in cannabis – could hold promise for treating prostate cancer. So does this mean that smoking…
August 19, 2009
In "Cancer in the news"

"Cannabis cure for brain cancer" headline is misleading
Read our in-depth post on cannabis and cannabinoids here Earlier this week the Daily Mail reported that a young US boy's brain tumour had been "cured" after his father secretly gave him cannabis oil through his feeding tube. The bold headline hides a more important truth: the boy was also…
May 5, 2011
In "Alternative/complementary"
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Kat Arney August 24, 2015
We've recently seen stories in the press claiming that the US government has "admitted that cannabis kills cancer" (for example, this one in the Metro), based on the observation that pages on the US National Cancer Institute information website carry details of the current scientific evidence around the effects of cannabis and cannabinoids on cancer cells in the lab and animal models.
The first thing to point out is that the NCI's cancer information website is an independent resource for doctors and the patients, and is not a statement of NIH, NCI or US government policy.
Furthermore, the information on these pages isn't new, nor is it an 'admission' of any kind: the scientific evidence about cannabis, cannabinoids and cancer, which these media stories are referring to, has been openly published on the NCI's website for several years – for example, see this page from the same section of the NIH website on cannabis and cannabinoids from 2011, accessed via the internet archive.

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Medicinal Cannabis and its Impact on Human Health
8092011
In this myth shattering, information packed documentary, learn from physicians and leading researchers about medicinal cannabis and its demonstrated affects on human health. This game-changing movie presents the most comprehensive synopsis to date of the real science surrounding the world's most controversial plant.
http://vimeo.com/20129106
Executive Producer: James Schmachtenberger
Director & Producer: Lindsey Ward
Director of Photography: Troy Brajkovich
Topics include:
*What the consensus is from over 15000 scientific and medical trials
*What conditions have been proven to benefit from medical marijuana
*Its historical use as medicine dating back over 5300 years
*Methods of delivery and their different advantages
*Government sponsored studies intended to show Marijuana having negative effects that yielded the exact opposite results
*Common myths about negative effects of Marijuana and what the research really says about these topics
Via: medicinalgenomics.com
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Tags: Common cannabis myths, controversial, medical marijuana, medical trials, scientific cannabis info
Categories : Cannabis Research, Cannabis Science, medicinal, Society and Cannabis

Canadian study shows relief for chronic neuropathic pain
31082010

There's now more scientific evidence for what many patients have known for awhile: Smoking marijuana can ease chronic neuropathic pain and help patients sleep better, according to a team of researchers in Montreal.
The new study, published Monday in the Canadian Medical Association Journal, found that pain intensity among patients decreased with higher-potency marijuana, reports Caroline Alphonso of The Globe and Mail. The study represents an important scientific attempt to determine the medicinal benefits of cannabis.
Patients suffering from neuropathic pain often use opioid pain medication, antidepressants and local anesthetics, but all of those drugs have limitations, and the side effects of these substances can rival the conditions they are supposed to treat. Unlike "normal" pain, which results from stimulation of pain receptors in the body, neuropathic pain results from damage to or dysfunction of the central or peripheral nervous system, reports Deborah Mitchell at EmaxHealth. Read the rest of this entry »
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Another resignation at the UK's Advisory Council on Drugs
14052010

Over the past 6 months the UK's Advisory Council on the Misuse of Drugs (ACMD)—an independent expert body that advises government on drug-related issues—has hardly been out of the headlines. One sacking and seven resignations is not a good track record for any organisation. The public's discontent at the ACMD over how it operates and how it is unduly influenced by government has left a bitter taste, together with a crisis in confidence about evidence-based policy making in the UK.
The trouble at the ACMD began in October, 2009, after the controversial sacking of the then chairman, Professor David Nutt for criticising the government's policy over cannabis and ecstasy. Five more members quit soon after in protest. In January, 2010, the equally distinguished neuroscientist, Professor Les Iverson, was appointed interim chair. In March, 2010, Dr Polly Taylor was the next to leave, outraged by the publication of the revised Code of Practice for Scientific Advisory Committees, the rewording of which compromised scientists' independence and would dissuade them from giving objective advice lest they disagreed with government policy.
The current outcry at the ACMD is over the recreational drug mephedrone (4-methylmethcathinone), a synthetic stimulant most similar chemically to amphetamines. It is a derivative of cathinone, a compound found in the plant called khat. Clinical and pharmacological research on cathinones is sparse and knowledge about the human effects of this drug class have been reliant on anecdotal reports from users and physicians. Adverse reactions include tachycardia, hallucinations, vasoconstriction, increased anxiety, and possible psychosis. The substance has received substantial media attention in the UK after reportedly being linked to 25 deaths. Indeed, the ACMD has suggested that media coverage has increased the use of the drug.
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Categories : Cannabis News, Cannabis Science, Society and Cannabis

Houses made of hemp could help combat climate change!
27102009

We have recently come across this very interesting press release from Professor Peter Walker at the University of Bath (U.K) who is leading the research into the use of hemp-lime in construction. Buildings and other infrastructure currently accounts for almost 20% of the UK's eco-footprint. This is another example of how this wonderful plant can help save reduce carbon dioxide emissions. Recently we brought you the news that Hanes – one of the worlds biggest consumer brands – has been investing in a new hemp technology called Crailar which requires only a fraction of the water needed to make cotton; and we are very happy to announce that it is the subject of another of our articles, a Dutch company called Hempflax who has won the contract to supply the raw materials to Hanes – i.e. the HEMP!
Here's the press release:
Houses made of hemp, timber or straw could help combat climate change by reducing the carbon footprint of building construction, according to researchers at the University of Bath.
Currently the construction industry is a major contributor of environmental pollutants, with buildings and other build infrastructure contributing to around 19% of the UK's eco-footprint. Researchers at the BRE Centre for Innovative Construction Materials are researching low carbon alternatives to building materials currently used by the construction industry. Although timber is used as a building material in many parts of the world, historically it is used less in the UK than in other countries. Researchers at the Centre are developing new ways of using timber and other crop-based materials such as hemp, natural fibre composites and straw bales. Their work using straw bales as a building material has already been featured on Channel 4's Grand Designs series.
Professor Peter Walker, Director of the Centre, is leading the research. He said: "The environmental impact of the construction industry is huge. For example, it is estimated that worldwide the manufacture of cement contributes up to ten per cent of all industrial carbon dioxide emissions. "We are looking at a variety of low carbon building materials including crop-based materials, innovative uses of traditional materials and developing low carbon cements and concretes to reduce impact of new infrastructure. As well as reducing the environmental footprint, many low carbon building materials offer other benefits, including healthier living through higher levels of thermal insulation and regulation of humidity levels."
Their research is being presented at the Sustainable Energy & the Environment showcase at the University of Bath. The exhibition will be opened by David Willetts MP, Shadow Secretary of State for Innovation, Universities & Skills, and will be attended by industrialists, research councils, local and national government representatives and other key stakeholders from across the South West. The exhibition coincides with the launch of the Institute for Sustainable Energy & the Environment (I-SEE) at the University of Bath, which will bring together experts from diverse fields of science, engineering, social policy and economics to tackle the problems of climate change.
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Hemp vs Cotton – The Pros and Cons

12102009
Historically, hemp has proven to be a versatile and durable substance and therein lies the contradiction. For nearly a century now, Hemp has been overlooked and under-appreciated as a viable alternative to cotton, possibly as a result of its connotative association with marijuana. To clarify, hemp has no psychoactive properties.

PROS: Hemp fibre is surprisingly useful as a cotton, paper, cellulosic or polymer substitute when it is treated correctly. As a crop, it is low-maintenance and resilient, and requires none of the weeding and heavy use of pesticides usually required in farming. Hemp is particularly effective as a source for textiles. The fibres drawn from the hemp plant are the strongest and longest in nature. Fabrics, twines, yarns and cords made from hemp are durable and versatile. It can be combed into any gauge or quality of fibre. As a substitute for such diverse substances as cotton, trees, or petroleum, hemp proves to be more environmentally sound than all of its alternatives (requiring about 10% of the water needed to produce cotton) and its versatility and resilience make it economically sound as well.
CONS: Hemp fibre is characterised by undesirable susceptibility to moisture and rot due to moulds and mildews and the like. It is also characterized by a strong, naturally-occurring odour which makes it unacceptable as a substitute for other odourless fibres. Moreover, hemp fibre in its natural spun state is susceptible to fraying and has a rough hand and feel. A need therefore remains for a hemp product which is suitably strong, soft, flexible, moisture-resistant and rot-resistant and generally suitable for substitution in applications previously focused on the cotton, paper and petroleum-fibre industries.
Hempflax are the leaders in this field in Europe and are based in Holland.
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Cannabis Patents – Cannabinoid Patch
12102009
United States Patent US6113940:
Cannabinoid patch and method for cannabis transdermal delivery.

We found this patent application in the U.S for a cannabis patch similar to the nicotine patch which has been commercially available for years. Several companies (and even the U.S government – see our previous article "U.S Government Patents Cannabis") have recently started to take notice of the medicinal properties of cannabis and have started a rush to patent any possible application of this wonderful natural herb. Part of this trend could be linked to the legalization movement which has successfully highlighted several medical advantages to cannabis over traditional pharmaceuticals, particularly the lack of significant negative side-effects when treating serious illness.
Here are the links to both the Patent outline and the Application in .pdf form
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Hanes invests in Hemp!
17082009
After a decade trying to prove that hemp could be soft as cotton, one American company is starting to draw the attention to its product from some of the world's biggest consumer brands.

Based in Portland, Oregon – Naturally Advanced Technologies Inc. – has developed an enzyme treatment which makes organic fibres (principally hemp and flax) suitable for producing clothes and other uses. This treatment is known as "Crailar Fiber Technology". Here's the information that Naturally Advanced provides about Crailar on their website:
"CRAILAR employs a simple, efficient 100% organic, enzyme bath and scales easily to leverage the global industrial hemp industry. In addition, CRAILAR Organic Fiber will be cost-comparable to organic cotton. Therefore, CRAILAR enables the transformation of hemp into a better sustainable alternative to organic cotton. "
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Cannabis as a cancer cure?
12082009

On the surface, it stands to reason that people who smoke cannabis could possibly face similar elevated risks for head and throat cancers as do those who smoke tobacco, and/or drink alcohol. Both alcohol and tobacco use carry significantly higher risks for such cancers.
In 1999, a highly – publicised study indicated that cannabis users might face some elevated risk of head and neck cancers, as is the case with consumers of tobacco and alcohol. However, even the researchers from that study cautioned against making too much of their results and called for further study.
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Medical marijuana may protect against swine flu!
11082009

As schools return from their summer breaks, there is widespread concern amongst parents and teachers alike. Swine Flu (H1N1) thrives off the conditions typical in classrooms and younger demographics are particularly vulnerable. As manufacturers struggle to meet unprecedented demand, one company has a controversial idea.
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Dr. Bob Melamede – Biology of Democracy
26062009

Dr. Robert Melamede aka Dr. Bob is a professor specializing in cancer treatment working at the university of Colorado.
In the video below Dr. Bob explains about Endocannabinoids and the difference between BLP's (Backward Looking People)and FLP's (Forward Looking People).
His theory on Endocannabinoids and how they influence everything is interesting. Have a look for yourself as he explains the basics in this video.
He also had some very interesting things to say about Cancer in a response to the Rick Simpson story (Run from the Cure), which we expect many of you have heard about. If you haven't, we recommend you watch it right now. Click here..
Want to know more? Have a look at Dr. Bob's youtube channel or his website.
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Tags: BLP, cancer treatment, Endocannabinoids, FLP, Rick Simpson, Robert Melamede, Run form the cure, university of Colorado
Categories : Cannabis Research, Cannabis Science, medicinal, Quotes & Opinions, Society and Cannabis

Marijuana good for your brain
26062009
Forget the stereotype about dopey potheads. It seems marijuana could be good for your brain.

While other studies have shown that periodic use of marijuana can cause memory loss and impair learning and a host of other health problems down the road, new research suggests the drug could have some benefits when administered regularly in a highly potent form.
Most "drugs of abuse" such as alcohol, heroin, cocaine and nicotine suppress growth of new brain cells. However, researchers found that cannabinoids promoted generation of new neurons in rats' hippocampuses.
Hippocampuses are the part of the brain responsible for learning and memory, and the study held true for either plant-derived or the synthetic version of cannabinoids.
"This is quite a surprise," said Xia Zhang, an associate professor with the Neuropsychiatry Research Unit at the University of Saskatchewan in Saskatoon.
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Tags: brain, cannabinoids, cannabis, health, Hippocampuses, HU-210, marijuana, medicinal, neurons, Neuropsychiatry Research Unit, receptor, THC, University of Saskatchewan, Xia Zhang
Categories : Cannabis News, Cannabis Research, Cannabis Science, medicinal

Top Anti-Drug Researcher says Legalize Marijuana
8062009
One of the world's foremost lung health experts says it's time to legalize marijuana.

Dr. Donald Tashkin, expert on marijuana and lung health, has called for the legalization of marijuana.Dr. Donald Tashkin, expert on marijuana and lung health, has called for the legalization of marijuana.For 30 years, Donald Tashkin has studied the effects of marijuana on lung function. His work has been funded by the vehemently anti-marijuana National Institute on Drug Abuse, which has long sought to demonstrate that marijuana causes lung cancer. After 3 decades of anti-drug research, here's what Tashkin has to say about marijuana laws:
"Early on, when our research appeared as if there would be a negative impact on lung health, I was opposed to legalization because I thought it would lead to increased use and that would lead to increased health effects," Tashkin says. "But at this point, I'd be in favor of legalization. I wouldn't encourage anybody to smoke any substances. But I don't think it should be stigmatized as an illegal substance. Tobacco smoking causes far more harm. And in terms of an intoxicant, alcohol causes far more harm."
We've been told a thousand times that marijuana destroys your lungs, that it's 5 times worse than cigarettes, and on and on. Yet here is Donald Tashkin, literally the top expert in the world when it comes to marijuana and lung health, telling us it's time to legalize marijuana. His views are shaped not by ideology, but rather by the 30 years he spent studying the issue. He didn't expect the science to come out in favor of marijuana, but that's what happened and he's willing to admit it.
Here's the study that really turned things around:
UCLA's Tashkin studied heavy marijuana smokers to determine whether the use led to increased risk of lung cancer and chronic obstructive pulmonary disease, or COPD. He hypothesized that there would be a definitive link between cancer and marijuana smoking, but the results proved otherwise."What we found instead was no association and even a suggestion of some protective effect," says Tashkin, whose research was the largest case-control study ever conducted.
Prejudice against marijuana and smoking in general runs so deep for many people that it just seems inconceivable that marijuana could actually reduce the risk of lung cancer. But that's what the data shows and it not only demolishes a major tenet of popular anti-pot propaganda, but also points towards a potentially groundbreaking opportunity to develop cancer cures through marijuana research.
Source: stopthedrugwar.org
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Tags: cancer cure, cannabis, cannabis prohibition, Donald Tashkin, Legalisation, lung cancer, lungs, marijuana, National Institute on Drug Abuse, research
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Cannabis cures Cancer?
31032009

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Tags: cancer, cannabis, cure, marijuana, medicinal, research, Robert Melamede, THC, weed
Categories : Cannabis News, Cannabis Research, Cannabis Science

Top Cannabis resources on the internet
9122008

We have compiled a list of what we feel are the top 8 online resources about cannabis on the internet. Every single one of these sites offers quality information on your favourite herb. There is so much information on this subject that even the most experienced canna-enthusiast can learn something new.
• erowid.org
Comprehensive website with lots of information cannabis. This website offers a lot of information on other drugs too. The link above is directly to the cannabis section of erowid.
• encod.org
The website of the European Coalition for just and effective drugs policies offers lots of information the drug policies of the various European countries with an emphasis on the policies on cannabis.
• marijuana-uses.com
A list of articles on Cannabis written by all sorts of people, academics and regular folks, with some very interesting views on this topic. Composed by Dr. Lester Greenspoon.
• thehia.org
The website of the Hemp Industries Association. A lot of info on the industrial applicability of Hemp. A comprehensive list of facts, research papers and frequently asked question about Hemp.
• beckleyfoundation.org
A NGO who have published soem very interesting research papers on drugs in general and Cannabis in particular. Try searching for cannabis in the search box at the top right of this site.
• cannabiscollege.com
A non profit organisation based in the heart of Amsterdam's Red light District. They recently celebrated their 10th anniversary and hopefully will be informing people about cannabis for many years to come.
• norml.org
A website fighting for Marijuana law reforms. With their blog and FAQ section they really have quite a large information databse on all kinds of marijuana related news and insights. Most of the material on this website has a political background.
• cannabis-med.org
German website with information on medicinal marijuana in six different languages.
If you feel that there are some other websites that deserve a place in this list or are simply also interesting to visit, don't be shy and send us a message or dump the link in the comments below.
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Kat Arney July 4, 2014
Additional information:
We often see websites with long lists of scientific papers claiming that cannabis is a "cure" for various cancers. However, when we look at the detail of the data and the experimental detail of the research, it becomes clear that although they may be interesting and build evidence to show that cannabinoids may one day bring benefits for cancer patients, they are far from being a cure.
The main point to realise is that virtually all these studies have been done in cancer cells grown in the lab or in animals. These are quite artificial systems and are much less complex than a real cancer growing in a patient.
For example, most experiments with cells grown in the lab use cancer cells that were originally taken from a tumour many years ago, but have been grown for a long time in the lab – known as cell lines. One problem with such cells is that they are all very similar on a genetic and molecular level, but we know that in real cancers, the cells can be very different from each other and respond in different ways to treatments. Also the usual way of testing cannabinoids in animals has been done by transplanting cancer cells (either mouse or human) into mice. Usually only a small number (5-20) will be used for each experiment.
There's growing evidence that these particular kinds of models (known as xenografts) aren't as good at suggesting a treatment could work, compared to more sophisticated genetically engineered animals, as they don't accurately represent the situation in real tumours. So although these kinds of experiments can point towards useful approaches, as well as revealing the underlying molecular 'nuts and bolts' of what's going on, they can't tell us if something will definitely treat cancer effectively and safely in human patients. They do not "prove that cannabis cures cancer", as the headlines would have us believe.
Put simply, Petri dishes are not people. Most chemicals that show promise in lab or animal experiments turn out not to work as well as hoped when tested in patients. These kinds of human studies, known as clinical trials, are the only way we can really know if a cancer treatment is effective.
There's more about clinical trials on our website: http://www.cancerresearchuk.org/cancer-help/trials/types-of-trials/
It's also important to think about what's being claimed when people use the word "cure". To most people, including us, this means that a cancer is completely treated and does not come back. When we look at the data in the papers listed below, none of them come close to showing these kinds of results. For the experiments involving cells grown in the lab, a proportion of the cells are killed or stop growing, but some of them carry on. Similarly in animal experiments, there is no data that shows a 100 per cent success rate for cannabinoids. For example, most mice treated with cannabinoids will still have tumours, although the cancers may be growing more slowly and spread less in some of them.
This isn't just true for cannabinoids – it's true for virtually all cancer drugs used today. Cancer is a very complex biological problem – there are hundreds of different types of cancer, each with important molecular and genetic differences. There's good evidence to show that every individual's cancer is as unique as they are, and that tumours can evolve and change within the body to become resistant to treatments.
We know that cancer drugs don't work for everyone all the time – that's why there's so much effort going on to find more effective treatments – but it's vital that doctors have a solid body of evidence showing how well the treatments they're using are likely to work. If you or someone you loved were going to take any kind of drug, would you be happy if it had only been tested in very high doses on cancer cell lines grown in the lab, or in mice injected with cancer cells? Or would you want to know that it had been trialled in large numbers of people, and there was good data on how effective it is, whether it's safe in the dose given, what the side effects are, and the proportion of people that can be expected to get better?
This kind of evidence can only come from a combination of lab studies leading to clinical trials. At the moment, while there are hundreds of interesting lab studies of cannabinoids (just some of which are included in the list below) there is only one clinical trial that has been published. So for now, cannabinoids, whether natural or synthetic, are a very long way from being what we would describe as a "cure" for any type of cancer.
We've looked at each of the papers in one of the commonly-seen lists (for example, here), and noted down the kinds of experiments they are. Many of them are available as open access papers, so it's possible to look at the data for yourself. Hopefully this is a useful explanation of the kind of scientific research that is currently ongoing into cannabinoids and cancer, and the process of gathering evidence to show whether a potential cancer therapy works.
Brain Cancer
http://www.nature.com/bjc/journal/v95/n2/abs/6603236a.html These are the only published results of any clinical trial we are aware of testing cannabis for cancer, which we cover in the blog post. Nine people with advanced, terminal glioblastoma multiforme – an aggressive brain tumour – were given highly purified tetrahydrocannabinol (THC) through a tube directly into their brain. Eight people's cancers showed some kind of response to the treatment, and one didn't respond at all. All the patients died within a year, as might be expected for people with cancer this advanced.
http://www.ncbi.nlm.nih.gov/pubmed/11479216 This is a study of a synthetic cannabinoid tested in mice that have had rat brain tumour cells from a cell line injected into them.
http://www.jneurosci.org/content/21/17/6475.abstract This paper isn't about cancer.
http://jpet.aspetjournals.org/content/308/3/838.abstract This is a study using human brain tumour cell lines grown in the lab.
http://mct.aacrjournals.org/content/10/1/90.abstract This is a study testing the combined effects of temozolomide, a drug used to treat brain tumours, with cannabinoids in brain tumour cells grown in the lab and also mice that have been transplanted with a human brain tumour cell line.
Mouth and Throat Cancer
http://www.ncbi.nlm.nih.gov/pubmed/20516734 This is a study testing the effects of cannabinoids on a mouth cancer cell line growing in the lab.
Breast Cancer

http://www.ncbi.nlm.nih.gov/pubmed/20859676 This is a study with human breast cancer cell lines grown in the lab and mice that have been transplanted with mouse mammary (breast) cancer cells from a cell line.
http://www.ncbi.nlm.nih.gov/pubmed/18025276 This is a study investigating human breast cancer cell lines grown in the lab.
http://www.ncbi.nlm.nih.gov/pubmed/21915267 This is a study looking at whether samples of human breast tumours carry the receptors for cannabinoids. The researchers also test the effects of a synthetic cannabinoid on human breast cancer cells lines grown in the lab, and in mice that have been transplanted with a breast cancer cell line.
http://jpet.aspetjournals.org/content/early/2006/05/25/jpet.106.105247.full.pdf+html This papers looks at the effects of cannabinoids on human breast cancer and leukaemia cell lines grown in the lab, and also on mice that have been transplanted with rat thyroid cells that have been infected with a virus to make them cancerous, or cells from a human breast cancer cell line.
http://www.molecular-cancer.com/content/9/1/196 This study investigates whether samples of human breast cancers carry cannabinoid receptors. The researchers also test cannabinoids (THC and a synthetic cannabinoid) in genetically modified mice that are prone to developing breast cancers.
http://www.ncbi.nlm.nih.gov/pubmed/22776349 This is a review of the research into cannabinoids and cancer that had been carried out up until 2012. It is not a research paper.
http://www.pnas.org/content/95/14/8375.full.pdf+html This paper looks at the effects of an endocannabinoid (anandamide, produced by the body) on human breast cancer cell lines grown in the lab.
Lung Cancer
http://www.ncbi.nlm.nih.gov/pubmed/22198381?dopt=Abstract This paper looks at the effects of cannabidiol (CBD) on human lung cancer cell lines, and also mice that have been transplanted with cells from a lung cancer line.
http://www.ncbi.nlm.nih.gov/pubmed/21097714?dopt=Abstract This paper looks at the effects of synthetic cannabinoids on human lung cancer cell lines grown in the lab, and on mice that have been transplanted with a human lung cancer cell line.
http://www.nature.com/onc/journal/v27/n3/abs/1210641a.html This paper looks at the effects of THC on human lung cancer cell lines grown in the lab, and on mice that have been transplanted with a human lung cancer cell line.
Uterine, Testicular, and Pancreatic Cancers
http://www.cancer.gov/cancertopics/pdq/cam/cannabis/healthprofessional/page4 This is an overview of some of the animal and cell experiments that have been done. It is not a research paper.
http://cancerres.aacrjournals.org/content/66/13/6748.abstract This study looked at the effects of THC and a synthetic cannabinoid on two pancreatic cancer cells lines grown in the lab, and also in mice that had been transplanted with cells from a pancreatic cancer line. The researchers also show that human pancreatic cancer samples contain cannabinoid receptors.
Prostate Cancer
http://www.ncbi.nlm.nih.gov/pubmed/12746841?dopt=Abstract This paper looks at the effects of anandamide on three different human prostate cancer cell lines.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3339795/?tool=pubmed This is a review of research about cannabinoids and prostate cancer. It is not a research paper.
http://www.ncbi.nlm.nih.gov/pubmed/22594963 This looks at the effects of non-THC cannabinoids (CBD and related compounds) on different human prostate cancer cell lines, and also on mice that have been transplanted with cells from two human prostate cancer lines.
Colorectal Cancer
http://www.ncbi.nlm.nih.gov/pubmed/22231745 This looks at whether CBD can reduce the incidence of bowel cancer in mice that have been treated with a chemical that causes tumours. The researchers also looked at bowel cancer cell lines grown in the lab.
Ovarian Cancer
http://www.aacrmeetingabstracts.org/cgi/content/abstract/2006/1/1084 This is an abstract from research that was presented at a meeting, so it's not possible to see the data or methods. From the abstract, it looks like the researchers have investigated the levels of cannabinoid receptors in human ovarian cancer cell lines grown in the lab, and also looked at the effects of a synthetic cannabinoid on these cell lines.
Blood Cancer
http://www.ncbi.nlm.nih.gov/pubmed/12091357 This paper looks at a range of mouse and human leukaemia and lymphoma cell lines grown in the lab, looking for the presence of cannabinoid receptors and their response to THC or a synthetic cannabinoid. They also test the effects of THC on mice that have been transplanted with mouse lymphoma cells. Two out of eight mice were still alive at the end of the experiment.
http://www.ncbi.nlm.nih.gov/pubmed/16908594 This paper looks at the effects of THC on a human leukaemia cell line grown in the lab, aiming to understand how the drug might be affecting the cells.
http://onlinelibrary.wiley.com/doi/10.1002/ijc.23584/abstract In this paper the researchers look at whether samples of Hodgkin's lymphoma from patients carry cannabinoid receptors. They also test the effects of a chemical based on anandamide in a human leukaemia cell line.
http://molpharm.aspetjournals.org/content/70/5/1612.abstract This looks at the effects of an anandamide-like chemical and synthetic cannabinoids on lymphoma cell lines and samples of lymphoma from patients. It's focusing on the molecular 'nuts and bolts' of what's going on in the cells rather than testing how well they work.
Skin Cancer
http://www.ncbi.nlm.nih.gov/pubmed/12511587 This paper looks at the effects of synthetic cannabinoids on mouse and human skin cancer cell lines grown in the lab, as well as mice that had been injected with mouse skin cancer cells – these were non-melanoma skin cancer cell lines, rather than melanoma. They also looked at the pattern of cannabinoid receptors in samples of human skin cancers.
Liver Cancer
http://www.ncbi.nlm.nih.gov/pubmed/21475304 This paper looks at the effects of THC and a synthetic cannabinoid on human liver cancer cell lines grown in the lab. They also looked at the effects of these drugs on mice that had been transplanted with cells from a human liver cancer line.
Biliary Tract Cancer
http://www.ncbi.nlm.nih.gov/pubmed/19916793 The link to this paper isn't working so we're unable to see the data or methods. From the abstract, it appears the researchers have tested THC on cholangiocarcinoma (bile duct cancer) cells grown in the lab, and also looked at patterns of cannabinoid receptors in samples of human bile duct tumours.
Bladder Cancer
http://www.medscape.com/viewarticle/803983 This is a report from a scientific meeting looking at whether smoking cannabis affects bladder cancer risk. The data have not been published in a scientific journal so it isn't possible to look at them in detail or draw any firm conclusions.
Cancer in General
http://www.ncbi.nlm.nih.gov/pubmed/12514108 This paper looks at the effects of cannabinoids on blood vessel growth in tumours. The researchers test a synthetic cannabinoid on mice that have been transplanted with brain tumour cells, looking in particular at the tumour blood vessels, and also look at the effects of cannabinoids on human blood vessel cells growing in the lab.
http://www.ncbi.nlm.nih.gov/pubmed/15313899 This paper looks at how activity levels of certain genes in mouse brain tumours change when given a synthetic cannabinoid, focusing on genes involved in the growth of new blood vessels. The researchers also look at the effects of cannabinoids on human brain tumour cells and other cancer cells growing in the lab, and at certain molecular markers in tumour samples taken from patients involved in the clinical trial described in the first paper in this list (Guzman et al http://www.nature.com/bjc/journal/v95/n2/abs/6603236a.html)

HEALTH CANNABIS
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Huntington's disease (HD) is a neurodegenerative genetic disorder that affects muscle coordination and leads to cognitive decline and behavioural symptoms.
HUNTINGTON'S DISEASE & Cannabis studies completed

1981 - Study ~ Tetrahydrocannabinol potentiates reserpine-induced hypokinesia.
1986 - Study - EFFECTS OF CANNABIDIOL IN HUNTINGTON'S DISEASE.
1993 - Study ~ Loss of cannabinoid receptors in the substantia nigra in Huntington's disease.
1999 - Study ~ Cannabis in movement disorders.
2000 - Study ~ Cannabinoid Receptor Messenger Rna Levels Decrease in a Subset of Neurons of the Lateral Striatum, Cortex and Hippocampus of Transgenic Huntington's Disease Mice.
2001 - Study ~ Changes in endocannabinoid transmission in the basal ganglia in a rat model of Huntington's disease.
2002 - Study ~ Alleviation of motor hyperactivity and neurochemical deficits by endocannabinoid uptake inhibition in a rat model of Huntington's disease.
2002 - Study ~ Loss of cannabinoid CB(1) receptors in the basal ganglia in the late akinetic phase of rats with experimental Huntington's disease.
2003 - Study ~ Compounds acting at the endocannabinoid and/or endovanilloid systems reduce hyperkinesia in a rat model of Huntington's disease.
2003 - Study ~ Effects of cannabinoids in the rat model of Huntington's disease generated by an intrastriatal injection of malonate.
2003 - Study ~ The endocannabinoid system and Huntington's disease.
2004 - Study ~ Structure, expression and regulation of the cannabinoid receptor gene (CB1) in Huntington's disease transgenic mice.
2004 - Study ~ Delayed onset of Huntington's disease in mice in an enriched environment correlates with delayed loss of cannabinoid CB1 receptors.
2005 - Study ~ Cannabinoid control of motor function at the basal ganglia.
2005 - Study ~ Abnormal sensitivity to cannabinoid receptor stimulation might contribute to altered gamma-aminobutyric acid transmission in the striatum of R6/2 Huntington's disease mice.
2006 - Study ~ UCM707, an inhibitor of the anandamide uptake, behaves as a symptom control agent in models of Huntington's disease and multiple sclerosis, but fails to delay/arrest the progression of different motor-related disorders.
2006 - Letter - Nabilone Could Treat Chorea and Irritability in Huntington's Disease.
2007 - Study ~ Cannabinoids and neuroprotection in motor-related disorders.
2008 - Study ~ Altered Lipid Metabolism in Brain Injury and Disorders.
2008 - Study ~ The endocannabinoid system in Huntington's disease.
2008 - Study ~ The endocannabinoid pathway in Huntington's disease: a comparison with other neurodegenerative diseases.
2008 - Study ~ Role of CB2 receptors in neuroprotective effects of cannabinoids.
2009 - Study - The endocannabinoid system as a target for the treatment of motor dysfunction.
2009 - Study ~ Microglial CB2 cannabinoid receptors are neuroprotective in Huntington's disease excitotoxicity.
2009 - Study ~ Altered CB1 receptor and endocannabinoid levels precede motor symptom onset in a transgenic mouse model of Huntington's disease.
2009 - Study ~ The endocannabinoid system as a target for the treatment of motor dysfunction.
2009 - Study ~ A pilot study using nabilone for symptomatic treatment in Huntington's disease.
2009 - Study ~ Cannabinoids and neurodegenerative diseases.
2009 - Study ~ Cannabinoid CB2 receptor agonists protect the striatum against malonate toxicity: relevance for Huntington's disease.
2009 - News ~ Medical Marijuana and Huntington's Disease.
2010 - Study ~ Widespread Decrease of Type 1 Cannabinoid Receptor Availability in Huntington Disease In Vivo.
2010 - Study ~ Cannabinoids and Dementia: A Review of Clinical and Preclinical Data.
2010 - Study ~ Enhancement of endocannabinoid signaling by fatty acid amide hydrolase inhibition: a neuroprotective therapeutic modality.
2010 - Study ~ Neuroprotective potential of CB1 receptor agonists in an in vitro model of Huntington's disease.
2010 - Study ~ Behavioural and molecular consequences of chronic cannabinoid treatment in Huntington's disease transgenic mice.
2011 - Study ~ Neuroprotective effects of phytocannabinoid-based medicines in experimental models of Huntington's disease.
2011 - Study ~ Prospects for cannabinoid therapies in basal ganglia disorders.
2011 - Study ~ Loss of striatal type 1 cannabinoid receptors is a key pathogenic factor in Huntington's disease.
2011 - Study ~ Worsening of Huntington disease phenotype in CB1 receptor knockout mice.
2011 - Study ~ Metabolic and Type 1 cannabinoid receptor imaging of a transgenic rat model in the early phase of Huntington disease.
2011 - Study ~ Unbalance of CB1 receptors expressed in GABAergic and glutamatergic neurons in a transgenic mouse model of Huntington's disease.
2012 - Study ~ Cannabinoids: Novel Medicines for the Treatment of Huntington's Disease.
2012 - Study ~ Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid?
2012 - Study ~ Cannabinoid modulation of neuroinflammatory disorders
2012 - Study ~ The dynamic nature of type 1 cannabinoid receptor (CB1) gene transcription
2012 - Study ~ The endocannabinoid system in normal and pathological brain ageing
2012 - Study ~ Sativex-like Combination of Phytocannabinoids is Neuroprotective in Malonate-Lesioned Rats, an Inflammatory Model of Huntington's Disease: Role of CB(1) and CB(2)
Receptors.
2012 - Study ~ Downregulation of cannabinoid receptor 1 from neuropeptide Y interneurons in the basal ganglia of patients with Huntington's disease and mouse models.
2012 - Study ~ Cannabinoid Receptor 2 Signaling in Peripheral Immune Cells Modulates Disease Onset and Severity in Mouse Models of Huntington's Disease.
2013 - Study ~ Cannabinoids increase type 1 cannabinoid receptor expression in a cell culture model of striatal neurons: implications for Huntington's disease.
2013 - Study ~ In vitro and in vivo models of Huntington's disease show alterations in the
endocannabinoid system.
2013 - Study ~ CNR1 variation is associated with the age at onset in Huntington disease.
2013 - Study ~ Therapeutic Potential of Cannabinoids in Neurodegenerative Disorders: A Selective Review.
2013 - Study ~ The Influence of Cannabinoids on Generic Traits of Neurodegeneration.
2012 - Study ~ The cytokine and endocannabinoid systems are co-regulated by NF-κB p65/RelA in cell culture and transgenic mouse models of Huntington's disease and in striatal tissue from
Huntington's disease patients.

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EFFECTS OF CANNABIDIOL IN HUNTINGTON'S DISEASE
Neurology 36 (Suppl 1) April 1986 p. 342
Reuven Sandyk, Paul Consroe, Lawrence Z. Stern, and Stuart R. Snider, Tucson, AZ

Cannabidiol (CBD) is a major nonpsychoactive cannabinoid of marijuana. Based on reports indicating possible efficacy of CBD in dystonic movements (Neurology 1984; 34 [Suppl 1]: 147 and 1985; 35 [Suppl 1]: 201), we tried CBD in three patients with Huntington's disease (HD).

The patients;, aged 30 to 56, had HD of 7 to 12 years' duration. Their condition has been slowly progressive and unresponsive to prior therapy with neuroleptics. Orally administered CBD was initiated at 300 mg/d and increased 1 week later to 600 mg/d for the next 3 weeks. Mild improvement (5 to 15%) in the choreic movements was documented using the tongueprotrusion test (Neurology [Minneap} 1972; 22: 929-33) and a chorea severity evaluation scale (Br J Clin Pharmacol 1981; 11: 129-51) after the first week.

Further improvement (20 to 40%) was noticed after the second week of CBD, and this remained stable for the following 2 weeks.

Except for transient, mild hypotension, no side effects were recorded, and laboratory tests were normal. Withdrawal of CBD after 48 hours resulted in return of choreic movements to the pre-CBD state.

(Supported in part by NINCDS grant #NS15441)

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Nabilone Could Treat Chorea and Irritability in Huntington's Disease
J Neuropsychiatry Clin Neurosci 18:553-554, November 2006
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Letter
Adrienne Curtis, B.A., B.Sc. and Hugh Rickards, M.D., M.R.C.Psych., Department of Psychiatry, University of Birmingham, Birmingham, United Kingdom
SIR: Huntington's disease causes chorea and psychiatric abnormalities. Psychiatric symptoms were found in one study in 51 out of 52 patients. Dysphoria, agitation, irritability, apathy, and anxiety were found in above 50% of the patients sampled.
Many sources postulate that cannabinoids could have a beneficial effect on the symptoms of Huntington's disease, especially on choreatic movements. As well as providing possible symptomatic relief in Huntington's disease, there is also some evidence that cannabinoids might have a neuroprotective effect which could delay the onset of symptoms by delaying or preventing the death of striatal neurons. This neuroprotective effect has also been postulated by other sources.
To date there are only two reports on the use of cannabinoids in Huntington's disease in the literature. Cannabidiol, a nonpsychotropic cannabinoid, had no effect on chorea severity in 15 patients. In one single patient, single dose, uncontrolled open clinical trial using nabilone, 1.5mg, the chorea increased significantly. We present a case of a female patient with irritability, which improved after the introduction of cannabis. This improvement was maintained by treatment with nabilone.
Case Report
The patient was a 43-year-old female who died in December 2003. She developed symptoms of Huntington's disease at the age of 24 and her husband gave up paid employment to care for her in 1990 when she was 30 years old. In 1995, he reported difficulties in caring for his wife. These difficulties were related to personality changes due to her illness.
She increasingly resisted help from professionals, especially care assistants, and refused any suggestion of short-term respite care. She became disinhibited and frequently undressed herself and walked around naked inside and occasionally outside the house. She exhibited a number of dangerous behaviors, such as leaving taps running and fires burning, and leaving burning cigarettes around. Her husband became concerned about the effect that the care for his chronically ill wife was having on his son, who was born in 1984.
The patient went into residential care in 1996. The patient's husband visited two or three times every week and he always took his wife out for a trip. These trips were difficult because of the patient's refusal to be strapped into the car or her wheelchair, which sometimes resulted in falls caused by violent choreic movements when he was unable to physically hold her in the chair because he was using his hands for some other purpose.
In 2001, he began to give his wife cannabis to smoke when he took her out on these regular trips. When he returned his wife to the nursing home after these visits the staff were aware of a significant difference in the patient. The cannabis appeared to improve her mood and she was calmer and more relaxed.
Prior to the introduction of cannabis she was extremely impatient and would get angry if required to wait even a few minutes for a cigarette. After taking cannabis, she was able to wait a while without screaming and throwing things. The patient also willingly accepted the use of a car seat belt and wheelchair harness.
In December 2001, the local general practitioner prescribed a regimen of nabilone, a synthetic 9-keto cannabinoid, which the patient began taking, 1mg each day. The husband and the nursing home staff both reported improvements in behavior and reduction of chorea coinciding with the

introduction of cannabis and maintained by daily taking nabilone.
Comment
This report has many limitations. It is a single case report and no measurements were taken at the time of the introduction of cannabis and nabilone. The information was obtained by interviewing the husband and staff from the care home in 2005.
The symptoms of Huntington's disease do change over time and the movements are different in the later stages of the disease. However both the husband and the staff are sure that the introduction of cannabis was beneficial and greatly improved the patient's quality of life in her last years. There is need for further trials to establish the therapeutic use of cannabinoids in the symptomatic treatment of Huntington's disease.
ACKNOWLEDGMENTS
The first author receives an unrestricted educational grant from Cambridge Laboratories, which holds the European marketing rights for nabilone.
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The endocannabinoid system as a target for the treatment of motor dysfunction

Br J Pharmacol. 2009 Apr;156(7):1029-40. Epub 2009 Feb 13
Fernández-Ruiz J.
Departamento de Bioquímica y Biología Molecular and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Facultad de Medicina, Universidad Complutense, Madrid, Spain.
Abstract
There is evidence that cannabinoid-based medicines that are selective for different targets in the cannabinoid signalling system (e.g. receptors, inactivation mechanism, enzymes) might be beneficial in basal ganglia disorders, namely Parkinson's disease (PD) and Huntington's disease (HD). These benefits not only include the alleviation of specific motor symptoms [e.g. choreic movements with cannabinoid receptor type 1 (CB(1))/transient receptor potential vanilloid type 1 agonists in HD; bradykinesia with CB(1) antagonists and tremor with CB(1) agonists in PD], but also the delay of disease progression due to the neuroprotective properties demonstrated for cannabinoids (e.g. CB(1) agonists reduce excitotoxicity; CB(2) agonists limit the toxicity of reactive microglia; and antioxidant cannabinoids attenuate oxidative damage).
In addition, extensive biochemical, anatomical, physiological and pharmacological studies have demonstrated that:
(i) the different elements of the cannabinoid system are abundant in basal ganglia structures and they are affected by these disorders;
(ii) the cannabinoid system plays a prominent role in basal ganglia function by modulating the neurotransmitters that operate in the basal ganglia circuits, both in healthy and pathological conditions; and (iii) the activation and/or inhibition of the cannabinoid system is associated with important motor responses that are maintained and even enhanced in conditions of malfunctioning and/or degeneration.
In this article we will review the available data regarding the relationship between the cannabinoid system and basal ganglia activity, both in healthy and pathological conditions and will also try to identify future lines of research expected to increase current knowledge about the potential therapeutic benefits of targeting this system in PD, HD and other basal ganglia disorders.
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19220290
[PubMed - indexed for MEDLINE]

PMCID: PMC2697699

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Natriuretic Peptides, Their Receptors, and Cyclic Guanosine Monophosphate-Dependent Signaling Functions
Lincoln R. Potter, Sarah Abbey-Hosch, and Deborah M. Dickey
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Abstract
Natriuretic peptides are a family of structurally related but genetically distinct hormones/paracrine factors that regulate blood volume, blood pressure, ventricular hypertrophy, pulmonary hypertension, fat metabolism, and long bone growth. The mammalian members are atrial natriuretic peptide, B-type natriuretic peptide, C-type natriuretic peptide, and possibly osteocrin/musclin. Three single membrane-spanning natriuretic peptide receptors (NPRs) have been identified. Two, NPR-A/GC-A/NPR1 and NPR-B/GC-B/NPR2, are transmembrane guanylyl cyclases, enzymes that catalyze the synthesis of cGMP. One, NPR-C/NPR3, lacks intrinsic enzymatic activity and controls the local concentrations of natriuretic peptides through constitutive receptor-mediated internalization and degradation. Single allele-inactivating mutations in the promoter of human NPR-A are associated with hypertension and heart failure, whereas homozygous inactivating mutations in human NPR-B cause a form of short-limbed dwarfism known as acromesomelic dysplasia type Maroteaux. The physiological effects of natriuretic peptides are elicited through three classes of cGMP binding proteins: cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels. In this comprehensive review, the structure, function, regulation, and biological consequences of natriuretic peptides and their associated signaling proteins are described.
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From <http://press.endocrine.org/doi/abs/10.1210/er.2005-0014>

The Emerging Role of the Endocannabinoid System in Endocrine Regulation and Energy Balance
Uberto Pagotto, Giovanni Marsicano, Daniela Cota, Beat Lutz, and Renato Pasquali
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Abstract
Section:

During the last few years, the endocannabinoid system has emerged as a highly relevant topic in the scientific community. Many different regulatory actions have been attributed to endocannabinoids, and their involvement in several pathophysiological conditions is under intense scrutiny. Cannabinoid receptors, named CB1 receptor and CB2 receptor, first discovered as the molecular targets of the psychotropic component of the plant Cannabis sativa, participate in the physiological modulation of many central and peripheral functions. CB2 receptor is mainly expressed in immune cells, whereas CB1 receptor is the most abundant G protein-coupled receptor expressed in the brain. CB1 receptor is expressed in the hypothalamus and the pituitary gland, and its activation is known to modulate all the endocrine hypothalamic-peripheral endocrine axes. An increasing amount of data highlights the role of the system in the stress response by influencing the hypothalamic-pituitary-adrenal axis and in the control of reproduction by modifying gonadotropin release, fertility, and sexual behavior.
The ability of the endocannabinoid system to control appetite, food intake, and energy balance has recently received great attention, particularly in the light of the different modes of action underlying these functions. The endocannabinoid system modulates rewarding properties of food by acting at specific mesolimbic areas in the brain. In the hypothalamus, CB1 receptor and endocannabinoids are integrated components of the networks controlling appetite and food intake. Interestingly, the endocannabinoid system was recently shown to control metabolic functions by acting on peripheral tissues, such as adipocytes, hepatocytes, the gastrointestinal tract, and, possibly, skeletal muscle. The relevance of the system is further strenghtened by the notion that drugs interfering with the activity of the endocannabinoid system are considered as promising candidates for the treatment of various diseases, including obesity.
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22. A. Emerging issues in the treatment of obesity and related diseases by cannabinoid antagonists
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24. VI. Summary and Perspectives

I. Introduction

Section:

THE FIRST STEPS in the discovery of the endocannabinoid system date back almost 4000 yr, when the therapeutic and psychotropic actions of the plant Cannabis sativa were first documented in India (1). Over the last 40 yr, after Gaoni and Mechoulam (2) purified the psychoactive component from hemp, a stunning amount of research has revealed the endocannabinoid system as a central modulatory system in animal physiology. Elements of the endocannabinoid system comprise the cannabinoid receptors, the endogenous lipid ligands (endocannabinoids), and the machinery for their biosynthesis and metabolism (3, 4). Despite public concern related to the abuse of marijuana and its derivatives, the research on the endocannabinoid system has recently aroused enormous interest not only for the physiological functions, but also for the promising therapeutic potentials of drugs interfering with the activity of cannabinoid receptors. This review aims to provide an overview on the pivotal role of the endocannabinoid system in the modulation of the neuroendocrine and peripheral endocrine systems. Moreover, in the context of the recently proposed therapeutic applications of cannabinoid receptor antagonists in the treatment of obesity, the key role of the endocannabinoid system in the control of eating behavior, food intake, and energy metabolism will be discussed in the light of the recent data obtained from human and animal studies.

II. The Endocannabinoid System

Section:

The large and widespread medical, religious, and recreational use of marijuana throughout the ages was apparently not sufficient to initiate careful and extensive research on cannabinoids until the last few decades of the 20th century. Conversely, the political antimarijuana attitude in the United States and the consequent prohibition in the 1930s did not help to encourage scientific interest on this topic. In the 1960s, the growing public concern regarding the potential negative healthy effects of cannabinoids associated with the exponential increase in its recreational use forced governmental institutions to invest resources to understand the modes of action of marijuana and the pathophysiological implications of its use in more detail. Cannabinoid research received a pivotal boost from the characterization of the chemical structure of Δ9-tetrahydrocannabinol (Δ9-THC), the main psychoactive constituent of marijuana (2). This finding paved the way to the understanding of marijuana's mechanisms of action and, many years later, to the cloning of the two receptor subtypes that are able to bind exogenous cannabinoids, named cannabinoid receptor type 1 (CB1 receptor) and type 2 (CB2 receptor), respectively, and to the identification of their endogenous ligands: the endocannabinoids (5, 6, 7, 8, 9). Cannabinoid receptors, endocannabinoids, and the machinery for their synthesis and degradation represent the elements of a novel endogenous signaling system (the so-called endocannabinoid system), which is involved in a plethora of physiological functions (3, 4). During the last few years, an overwhelming amount of data has been acquired to understand the biological roles of this system in more detail. However, many questions are still open, and promising new discoveries await us in the near future.
In general, the endocannabinoid system is involved in many different physiological functions, many of which relate to stress-recovery systems and to the maintenance of homeostatic balance (10). Among other functions, the endocannabinoid system is involved in neuroprotection (11, 12, 13), modulation of nociception (14), regulation of motor activity (15), and the control of certain phases of memory processing (16, 17, 18). In addition, the endocannabinoid system is involved in modulating the immune and inflammatory responses (19, 20, 21). It also influences the cardiovascular and respiratory systems by controlling heart rate, blood pressure, and bronchial functions (22). Finally, yet importantly, endocannabinoids are known to exert important antiproliferative actions in tumor cells (23). A full discussion of the plethora of functions of the endocannabinoid system in maintaining homeostasis is beyond the scope and space of the present review. However, the reviews cited in this article will further help to obtain a broad insight into the physiological roles of the endocannabinoid system.

A. Cannabinoid receptors

Two cannabinoid receptors have been identified and molecularly characterized so far, namely the seven transmembrane G protein-coupled cannabinoid receptor type 1 (CB1 receptor) (6) and type 2 (CB2 receptor) (7). CB1 receptor was originally described as the “brain type” cannabinoid receptor, because its levels of expression were high in the brain (24). However, recent studies attribute new sites of action of endocannabinoids to many peripheral organs through CB1 receptor activation. The generalization for CB1 receptor being the eminent “brain type” receptor is therefore no longer appropriate. Conversely, CB2 receptors are present almost exclusively in immune and blood cells, where they may participate in regulating immune responses (25). However, CB2 receptors also exert functions in nonimmune cells such as keratinocytes (26). Pharmacological evidence exists for the presence of other cannabinoid receptors, which, however, have not yet been cloned (27). The endocannabinoid anandamide is also able to bind to and activate vanilloid receptors, transient receptor potential vanilloid type 1 (28), and to inhibit TASK-1 K+ channels (29). Moreover, pharmacological studies indicate that still unidentified additional cannabinoid receptors might exist in the hippocampus, modulating the release of glutamate (30), and on endothelial cells (31). Two patents have been recently published claiming that a number of cannabinoid ligands also bind to GPR55, an orphan G protein-coupled receptor, suggesting that this receptor might represent a novel target of cannabinoid action (32). CB1 receptor, however, is the best characterized target of exogenous and endogenous cannabinoids in the modulation of neuroendocrine and metabolic responses, and this review will focus mainly on this receptor.

1. CB1 receptor expression in the brain.

Cannabinoid receptor distribution was studied by means of autoradiography of ligand-receptor binding on slide-mounted rat brain sections (24, 33), by in situ hybridization (ISH) (34, 35, 36), by autoradiography in human brain (37), by immunohistochemistry (IHC) (38, 39, 40, 41), and by agonist-stimulated [35S]GTPγS binding to slide-mounted sections (42, 43). Expression studies showed very early that CB1 receptor is one of the most abundant G protein-coupled receptors in the mammalian brain (24). CB1 receptors are widely expressed in the brain, including the olfactory bulb, cortical regions (neocortex, pyriform cortex, hippocampus, and amygdala), several parts of basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex, and brainstem nuclei. The levels of expression vary considerably among the various brain regions and neuronal subpopulations. For instance, agonist-mediated receptor binding revealed high densities of CB1 receptor protein in the cornu ammonis pyramidal cell layers of the hippocampus (24), which was later shown by IHC to be due to a dense plexus of immunoreactive fibers deriving from γ-aminobutyric acid (GABA)-ergic interneurons and surrounding the cell bodies of pyramidal cells, which appear per se to be devoid of CB1 receptor protein (38, 41, 44). However, pyramidal cells of the hippocampus and other cortical regions do express low but significant levels of CB1 receptor mRNA (34, 36), indicating the possibility that CB1 receptor protein in these cells is localized on distal projections and/or is expressed at low levels, which are below the limit of detectability with currently available immunohistochemical methods. A similar situation is present also in other cortical regions, such as the amygdala, neocortex, entorhinal cortex, and piriform cortex.

In subcortical regions, CB1 receptor is present at relatively high levels in the septal region (lateral and medial septum, and vertical and horizontal nuclei of the diagonal band). Lower levels of expression are present in hypothalamic regions, such as the medial and lateral preoptic nucleus, magnocellular preoptic nucleus, and paraventricular nucleus (PVN) (36). In the caudal hypothalamus, CB1 receptor is expressed in the premammillary nucleus. In the lateral hypothalamus, CB1 receptor is present in scattered cells (34, 36). In the PVN, CB1 receptor mRNA coexpresses with CRH mRNA (45). In the thalamus, CB1 receptor is present in the lateral habenula, reticular thalamic nucleus, and zona incerta. Midbrain dopaminergic neurons are generally considered to lack CB1 receptor expression. However, recent observations indicate that very low levels of CB1 receptor might be present in tyrosine hydroxylase-expressing neurons in the ventral tegmental area (VTA) (46) and in dopaminergic terminals in the striatum (47). In the hindbrain, apart from the molecular and granular layers of cerebellum expressing high levels of the receptor, CB1 receptor is present at low levels in some nuclei of the brain stem, such as the periaqueductal gray (34, 38). Functional mapping by agonist-stimulated [35S]GTPγS binding using different CB1 receptor agonists revealed that cannabinoid activation of G proteins occurs with the same regional distribution as the receptors (43, 48). However, in some regions, the ratio between the estimated amount of CB1 receptor and G protein activation is not always constant, thus indicating regional differences in receptor-coupling efficiencies (43). This is important to consider, because sometimes the endocannabinoid system seems to influence functions involving regions where the density of CB1 receptor is relatively low (e.g., modulation of food intake in the hypothalamic area). Therefore, the activity of cannabinoids on CB1 receptor cannot be predicted based solely on the relative receptor density, but other factors, such as receptor coupling efficiency, should be taken into account. For instance, by using conditional mutagenesis in mice, the relatively low levels of CB1 receptor expression in cortical pyramidal neurons were recently shown to play a central role in the endocannabinoid-mediated protection against excitotoxic seizures (12). In conclusion, CB1 receptor is widely expressed in the brain and is present at different levels in different neuronal subpopulation and brain regions, and there is apparently no strict correlation between levels of expression and receptor functionality.

2. CB1 receptor expression in the pituitary.

Early studies showed a scattered presence of CB1 receptor in both lobes of the rodent pituitary (33). Recent studies examined the distribution of CB1 receptor mRNA in the anterior pituitary lobe in more detail. In 1999, the abundant CB1 receptor presence in the rat adenohypophysis was associated with the ability of this gland to synthesize endocannabinoids (49). CB1 receptor was also shown to be present in prolactin (PRL)- and LH-secreting cells of the rat pituitary (50). CB1 receptor expression was also detected by means of double-immunofluorescence in the pituitary gland of Xenopus laevis, where the receptor was found in lactotrophs, gonadotrophs, and thyrotrophs (51). The expression of CB1 receptor in the human pituitary appears to be substantially different from the localization of the same receptor described in rodents and frogs (52). By using ISH and double IHC, CB1 receptor was localized in the majority of corticotrophs and somatotrophs of the normal human anterior lobe; only a small percentage of the PRL-secreting cells are positive for CB1 receptor, whereas no immunoreactivity was found in LH-, FSH-, or TSH-positive cells. The neural lobe is devoid of CB1 receptor immunoreactivity (52). Interestingly, folliculo-stellate cells are also positive for CB1 receptor, although functional data have not yet been associated with this expression (52). CB1 receptor was also found in human pituitary adenomas, such as ACTH-producing adenomas (which give rise to Cushing’s syndrome), GH-producing tumors (leading to acromegaly), and in prolactinomas, whereas no CB1 receptor staining was found in so-called nonfunctioning pituitary adenomas, tumors expressing LH and/or FSH, and/or α-subunit being devoid of any hormonal staining (52). These data were confirmed by a study in which cDNA microarray analysis was used to compare gene expression pattern in pituitary adenomas vs. normal pituitary (53). Among other genes differentially expressed, ACTH- and GH-producing tumors express higher levels of CB1 receptor compared with the normal pituitary (53). Notably, the human normal anterior pituitary gland and pituitary tumors were shown to be capable of synthesizing endocannabinoids (52).

In rodents, CB1 receptor expression in the pituitary is under the influence of circulating sex hormones, as demonstrated by the ability of androgens and estrogens to up- and down-regulate CB1 receptor, respectively (49). In agreement with these findings, decreased CB1 receptor expression has been found in estrogen-induced pituitary hyperplasia in rats (49). Accordingly, in rats, the male pituitary displays higher levels of CB1 receptor mRNA than the female one (49). In contrast, the human pituitary does not show this gender difference (52).

Exogenous cannabinoids can modulate the expression of CB1 receptor in the pituitary. After a transient down-regulation of the receptor (first 1–3 d), chronic administration of CB1 receptor agonists is able to produce a consistent increase of CB1 receptor expression in the anterior pituitary lobe (after 14 d) (54). This finding seems to be in contrast with the level in the ventromedial hypothalamic nucleus, where CB1 receptor mRNA was down-regulated by chronic CB1 receptor agonist treatment (54).

3. CB1 receptor expression in the peripheral organs

a. CB1 receptor in the thyroid gland.

CB1 receptor expression during the late embryological stages of the rat thyroid was found to be very high (55), whereas lower but still detectable levels of CB1 receptor mRNA and protein were present in the adult rat gland distributed in both follicular and parafollicular cells as demonstrated by IHC (56).

b. CB1 receptor in the adrenal gland.

A faint signal for CB1 receptor was detected in the human adrenal glands by quantitative RT-PCR method (57). However, ISH or IHC studies are needed to clearly localize CB1 receptor in the different areas that make up the gland.

c. CB1 receptor in the peripheral organs involved in metabolic control.
In 2003, two independent groups found the presence of CB1 receptor in adipocytes of mice and humans (58, 59, 60). In both species, this expression is more evident in mature adipocytes than in preadipocytes (59, 60), indicating that the full cellular machinery of the fat cell is needed to exert cannabinoid action. Little is known about CB1 receptor expression in the muscle. Recently, the CB1 receptor antagonist SR141716 was shown to directly affect glucose uptake in the isolated soleus muscle of genetically obese mice (61). Consistently, CB1 receptor is present in the murine soleus muscle as shown by RT-PCR (Fig. 1). Additional investigations are needed to fully understand the importance of this expression site.
Recently, an elegant study by Kunos' group (62) localized CB1 receptor in the mouse liver. CB1 receptor mRNA was detected by ISH with strong labeling in Kupffer cells, whereas lower levels of expression were found in hepatocytes and endothelial cells. Interestingly, CB1 receptor expression was more prominent in hepatocytes surrounding the central veins. Human hepatic stellate cells also have been shown to express CB1 receptor (63). At present, nothing is known about CB1 receptor in the exocrine and endocrine cells of the pancreas.
d. CB1 in the gastrointestinal tract.
The endocannabinoid system is present in the gastrointestinal tract where it modulates several functions, including motility, inflammation, and secretion (64). Interestingly, CB1 receptor is expressed in vagal nerve terminals innervating the gastrointestinal tract (64), which are involved in gut-brain signaling, modulating food intake. They express cholecystokinin (CCK) receptor type 1 whose activation is known to play a very important role in mediating satiety. Vagal neurons are known to express receptors for leptin and orexin-A (65, 66), whose ligands activate and reduce the anorectic effect of CCK on vagal afferent nerve discharge, respectively. Importantly, CB1 receptor is also present in these neurons, and its expression is decreased after feeding and enhanced in fasting conditions (67). CCK was shown to mediate the effect of food in down-regulating vagal CB1 receptor expression (67). CB1 receptor was also found in the fundus of the stomach, but the cellular localization is not yet known. However, a single SR141716 administration is able to reduce the levels of ghrelin (68), whose production takes place in the gastric endocrine (X-) cells (69).
e. CB1 receptor in the reproductive organs.
CB1 receptor has been known for a long time to be expressed in the testis (57, 70). In particular, it seems to be localized in Leydig cells (71), whereas Sertoli cells that are able to inactivate arachidonoyl ethanolamide (AEA) do not express CB1 receptor (72). Sea urchin sperms, an ideal model for studying fertilization processes, express cannabinoid binding sites (73). Human sperms possess functional binding sites for cannabinoids (74). Very recently, Rossato et al. (75) elegantly showed that CB1 receptor is present in the head and the middle piece of human sperm.
CB1 receptor is also expressed in the ovary (57), probably located in the granulosa cell layer where Δ9-THC was shown to inhibit cAMP accumulation (76). CB1 receptor is present in the mouse uterus (77) and in the human myometrium (78), and is associated with the relaxant effect of cannabinoid receptor agonists (78). Importantly, CB1 receptor is coexpressed with β-adrenergic receptors in the oviduct muscularis, where the endocannabinoid system regulates motility and embryo transport (79). Both CB1 and CB2 receptors are located in the mouse preimplantation embryos (80) as well as in all layers of human placenta; particularly high levels are detectable in the amniotic epithelium and in the maternal decidua layer (81).
4. Signal transduction of CB1 receptor.
The signal transduction of cannabinoid receptors has been extensively described in many excellent reviews (3, 4, 25, 82, 83, 84, 85), and its detailed description is beyond the scope of the present article. It is important to note, however, that CB1 receptor activation might lead to the stimulation of different intracellular pathways, depending on the cell type involved and the experimental conditions. For instance, CB1 receptor, which normally inhibits adenylate cyclase, can also stimulate the cAMP pathway in particular conditions (86, 87). Moreover, recent results suggest the possibility of functional interactions of CB1 receptors with other receptors, for instance, with type 1 orexin receptors (88), 5HT2 serotonin receptors (89), and dopamine receptor type 2 (D2) (87). The possibility that such interactions depend on heterooligomerization processes might represent a very interesting novel aspect (87), which will expand the view of the pharmacology and physiology of the endocannabinoid system. These considerations should also be borne in mind to understand the roles of the endocannabinoid system in regulating the endocrine systems. Figure 2 summarizes the best-described intracellular effects of CB1 receptor stimulation, including the regulation of the cAMP cascade, modulation of ion channels, stimulation of kinase pathways, and induction of immediate early genes.
B. Endocannabinoids
1. Structure.
In 1992, the first endogenous cannabinoid, AEA, also called anandamide, was identified (8). Subsequently, a second endocannabinoid, 2-arachidonoyl glycerol (2-AG), was discovered (5, 9). Both these compounds are derivatives of arachidonic acid and are able to bind to CB1 and CB2 receptors, although with differences in affinities and activation efficacies (90). During the last few years, several other bioactive lipid mediators have been described; they appear to act, at least in part, through CB1 and/or CB2 receptors and confer specific pharmacological effects in vivo (91). Specifically, these compounds are 2-arachidonoyl-glyceryl-ether (noladin ether) (92), O-arachidonoyl-ethanolamine (virodhamine) (93), N-arachidonoyl-dopamine (94), and possibly oleamide (95). However, the endogenous function in physiological processes for all these latter compounds have not yet been established in detail and need further investigation (4). Furthermore, there are several additional putative lipid mediators that might have cannabimimetic actions, but whose exact mechanism of action is not known in detail (91). In some cases, their cannabimimetic effects may be partially attributed to interference with the endocannabinoid-inactivating enzymes (91). These lipids might, therefore, be able to enhance the activity of cannabinoid receptors by increasing the concentration of the endocannabinoids such as AEA and/or 2-AG.
2. Synthesis, release, uptake, and degradation of endocannabinoids: on demand activation of the endocannabinoid system.
Endocannabinoids are very lipophilic and thus cannot be stored in vesicles like other neurotransmitters. Consequently, the regulation of endocannabinoid signaling is tightly controlled by their synthesis, release, uptake, and degradation (3). Several different stimuli, including membrane depolarization and increased intracellular Ca2+ and/or receptor stimulation, can activate complex enzymatic machineries, which lead to the cleavage of membrane phospholipids and eventually to the synthesis of endocannabinoids. Importantly, different enzymes are involved in the synthesis of distinct endocannabinoids, indicating an independent involvement of endocannabinoids in different conditions. After synthesis, endocannabinoids can activate cannabinoid receptors, either after previous release into the extracellular space or directly moving within the cell membrane.
Endocannabinoid signaling is limited by very efficient degradation processes, involving facilitated uptake from the extracellular space into the cell and enzymatic catabolism mediated by specific intracellular enzymes. The molecular nature of the carrier protein(s) involved in endocannabinoid uptake has not yet been elucidated. However, the enzymes able to degrade endocannabinoids are quite well characterized. They are fatty acid amide hydrolase (FAAH) for anandamide and related compounds (96) and monoglycerol lipase for 2-AG (97), although other enzymes might be partially involved in the degradation of this last compound (98). A detailed description of the biochemical mechanisms leading to the synthesis, release, uptake, and degradation of endocannabinoids is beyond the scope of the present article, and we refer the reader to several excellent and exhaustive reviews recently published on the subject (3, 4, 30, 82, 99, 100, 101). An interesting aspect of endocannabinoid activity is the rapid induction of their synthesis, receptor activation, and degradation (3, 102). The endocannabinoid system has thus been suggested to act on demand, with a tightly regulated spatial and temporal selectivity. The system exerts its modulatory actions only when and where it is needed. This fact poses an important distinction between the physiological functions of the endocannabinoid system (selective in time and space) and the pharmacological actions of exogenous cannabinoid receptor agonists, which lack such selectivity. In the context of endocrine regulation, it is interesting to mention here that hormonal stimulation with glucocorticoids can lead to the synthesis of endocannabinoids in the hypothalamus through rapid nongenomic mechanisms (103). It was also recently shown that phospholipase Cβ represents an intracellular coincidence detector of membrane depolarization and receptor stimulation leading to the synthesis and, possibly, the release of endocannabinoids in the hippocampus (104). These data reveal a novel mechanism for activation of the endocannabinoid system, which could also be involved in the regulation of endocrine systems. Concerning degradation of endocannabinoids, which represents an important regulatory aspect of the activity of the endocannabinoid system, it should also be mentioned that a recent study investigated whether endocytic processes are involved in the uptake of endocannabinoids and found that about half of the AEA uptake occurs via a caveola/lipid raft-related process (105).
3. Endocannabinoid-mediated inter- and intracellular signaling.

Several mechanisms underlying endocannabinoid-mediated signaling have been reported. 1) In the central nervous system (CNS), endocannabinoids can act as neurotransmitters transferring information from one neuron to the next. Here, postsynaptically released endocannabinoids travel to the presynaptic site where they activate CB1 receptors. They thus mediate a retrograde signal (30, 106, 107). The overall effect is a decrease in the release of neurotransmitters such as glutamate and GABA. This phenomenon is present in synaptic connections of many brain regions, thus representing an important modulatory mechanism of neuronal transmission. With respect to the aims of the present review, it is noteworthy that this function has also been shown in the VTA (108, 109), where the modulation of reward properties of food presumably occur, and in the hypothalamus, where endocannabinoids and CB1 receptor mediate the acute glucocorticoid-dependent depression of glutamatergic transmission (103). 2) Endocannabinoids can mediate an autocrine signaling that induces a self-inhibitory effect on neuronal activity. This was shown for GABAergic neurons in the cerebral cortex (110). 3) Endocannabinoids may act in a paracrine or autocrine manner, not involving synaptic transmission. This is presumably applicable for glial cells (111) and in nonneuronal cells such as the adipocytes and the hepatocytes. 4) Because endocannabinoids and CB1 receptor are also present within the cell, it cannot be excluded that endocannabinoids may act as intracellular signaling molecules. Importantly, AEA and 2-AG do not appear as interchangeable mediators. For instance, electrophysiological and biochemical evidence shows that 2-AG is mostly involved in retrograde control of synaptic activity in the VTA (109), or the hippocampus (112), whereas AEA appears to play an important role in other regions, such as the basal ganglia (113) and the amygdala (114).

In summary, endocannabinoids appear to be very versatile signaling mediators, involved in a broad spectrum of physiological regulatory processes.

C. Cannabinoid agonists

1. Plant-derived cannabinoids.

The isolation and characterization of the psychoactive component of C. sativa represented a challenging research task. This was due to the fact that the extracts from Cannabis plants contain more than 60 different, chemically closely related terpeno-phenols that are difficult to separate and purify. This prevented the isolation of pure crystals for determination of the structure. The breakthrough was achieved using improved column chromatography. As mentioned above, in the early 1960s, Gaoni and Mechoulam (2) succeeded in isolating and pharmacologically characterizing various plant-derived cannabinoids. In hemp, the major psychoactive compound is represented by Δ9-THC, whereas Δ8-tetrahydrocannabinol is only present in very low amounts. The majority of terpeno-phenols in hemp lack psychoactivity. They include cannabidiol, cannabinol, cannabigerol, and cannabichromene. Although psychoactive cannabinoids bind to and activate both CB1 and CB2 cannabinoid receptors, nonpsychoactive cannabinoids are also able to exert various pharmacological effects in vivo, although only at rather high concentrations and not by activation of CB1 or CB2 receptors. Cannabidiol has recently gained additional attention due to its anticonvulsive, neuroprotective, and antiemetic activities (115, 116, 117). The underlying mechanisms of actions of this plant-derived cannabinoid have not yet been elucidated.

2. Classification of exogenous and endogenous cannabinoids.

Based on structural features, plant-derived and synthetic cannabinoids are divided into different classes (25). In brief: 1) For “classic” cannabinoids, the main psychoactive constituent of Cannabis, Δ9-THC, encompasses tricyclic dibenzopyran compounds and serves as the lead structure. Δ9-THC is a partial agonist of CB1 and CB2 receptors. The synthetic derivative HU210 shows the highest potency among the known CB1 receptor agonists and also activates CB2 receptors (25). HU308, another synthetic Δ9-THC derivative, was found to be a selective CB2 receptor agonist (118). 2) So-called “nonclassic” cannabinoids are synthetic Δ9-THC derivatives that lack the dihydropyran ring. The most famous one is represented by CP-55,940, a potent and complete agonist of CB1 and CB2 receptors, synthesized by Pfizer. It was originally pivotal for the molecular identification of CB1 receptor (25). 3) Finally, aminoalkylindoles, represented by R-(+)-WIN-55,212-2, are compounds structurally unrelated to Δ9-THC but with strong cannabimimetic activities (25). They bind to both CB1 and CB2 receptors (25).

All endocannabinoids are structurally rather distinct from plant-derived and most synthetic cannabinoids. Prototypically, they belong to the eicosanoids, fatty acid derivatives containing a chain with 20 carbon atoms. The synthetic AEA derivative arachidonyl-2′-chloroethylamide represents a selective CB1 receptor agonist with very low activity on CB2 receptor (25).

The quest for specific ligands for either of the cannabinoid receptors represents an important research topic. In particular, if CB2 receptor is targeted with a specific agonist, with no activity on CB1 receptor, the psychotropic side effects of the agonist are avoided. This may be very relevant for alleviating peripheral pain where CB2 receptor is involved (26, 119). Further important progress may also be achieved by the development of cannabinoid receptor agonists that do not pass the blood-brain barrier. Such compounds would focus on the receptors in the periphery and would thus prevent undesirable side effects originating from the CNS.

Although not acting as ligands of cannabinoid receptors, inhibitors of cellular uptake of endocannabinoids, such as AM404 (120), VDM11 (121), and UCM707 (122) provide another interesting class of drugs interfering with the endocannabinoid system. Given the on demand nature of the synthesis and release of endocannabinoids, these drugs make it possible to induce a targeted increase in the concentration of endocannabinoids, likely reducing some of the undesirable side effects observed by using receptor agonists.

D. Cannabinoid type 1 receptor antagonists

Pharmacological investigations have placed emphasis on the generation of substances acting as specific antagonists of cannabinoid receptors. Among the increasing number of compounds sharing CB1 receptor antagonistic properties (123, 124), the compounds most characterized are SR141716 (125), SR14778 (126), AM251 (124), AM281 (127), LY320135 (128), and SLV319 (129). The CB1 receptor antagonists known so far are diarylpyrazoles, or aminoalkylindoles, or triazole derivatives. Diarylpyrazoles include SR141716, which is the first selective CB1 receptor antagonist reported. It was discovered approximately a decade ago, and it has been the compound most studied so far. Pharmacologically, SR141716 shows a Ki value of binding to rat brain synaptosome of 1.98 ± 0.36 nm (125). Few data on the metabolism and pharmacokinetics of SR141716 are available in humans (130). The dose of SR141716 that produced a 50% antagonism of agonist effect in the mouse was 0.23 mg/kg, and a dose of 3 mg/kg produces a long-lasting (18 h) blockade of the effect of WIN-55212–3 (131).

There are different possible mechanisms by which CB1 receptor antagonists produce their effects on the CB1 receptor (132). The ligands can be competitive antagonists of CB1 receptor activation by endogenously released endocannabinoids, or they can act as inverse agonists and modulating constitutive CB1 receptor activity by shifting it from an active “on” to an inactive “off” state (133). They may also act by CB1 receptor independent mechanisms (132). These mechanisms are not mutually exclusive.

III. Exogenous and Endogenous Cannabinoids and Their Role in Endocrine Regulation

Section:

It has been known for a long time that exogenous cannabinoids are able to affect secretion of pituitary hormones, thus having a strong effect on peripheral target organ functions. Notably, in 1972 the first report of an induction of gynecomastia due to marijuana consumption led to a dramatic acceleration of studies on this topic (134). The hypothalamus is generally considered as the main site of cannabinoid action on neuroendocrine functions. This view is elegantly supported by a recent publication showing that endocannabinoids act as retrograde messengers activating CB1 receptors expressed at presynaptic glutamatergic terminals in the hypothalamus (103). The subsequent activation of the CB1 receptor signaling cascade leads to the inhibition of the release of the excitatory neurotransmitter glutamate onto the neuroendocrine cells of the PVN and the supraoptic nucleus (103). This leads to a general suppressive effect on neuroendocrine cells and a final inhibitory effect on neuroendocrine function.

However, it was recently proposed that the endocannabinoid system might control hormonal balance also through a direct effect at the level of the peripheral target organs. An overview of the cannabinoid actions on endocrine axes is given in Table 1.

A. Cannabinoids and the hypothalamic-pituitary-adrenal axis

Stimulation of the hypothalamic-pituitary-adrenal (HPA) axis is a crucial neuroendocrine response to stress. Psychological or physiological stressors are known to induce CRH production in the PVN of the hypothalamus, eventually leading to a release of this hypothalamic peptide onto the anterior pituitary gland. In turn, this leads to increased circulating levels of ACTH and, finally, to an increase of corticosteroids secreted by the adrenal gland. Until a few years ago, the impact of the cannabinoids on the HPA axis was considered as an exception. Whereas the commonly accepted view attributes the cannabinoid system with a general inhibitory role on neuroendocrine functions, it was suggested that cannabinoids are, on the contrary,

able to stimulate the HPA axis. In fact, many studies in animals point to a CB1 receptor-dependent (135) increase of circulating ACTH and glucocorticoid levels after pharmacological administration of plant-derived (136), synthetic (137, 138), or endogenous cannabinoid agonists (139, 140). In agreement with this, a simultaneous elevation of CRH in the PVN and of proopiomelanocortin in the anterior pituitary after chronic treatment (18 d) with the CB1 receptor agonist CP-55,940 was observed in rats (138). Cannabinoids were proposed to act exclusively at hypothalamic sites after the finding that Δ9-THC did not induce hyperactivation of the HPA axis in hypophysectomized rats (141), and that Δ9-THC or WIN 55,212-2 was unable to stimulate ACTH release from basal and CRH-stimulated dispersed pituitary cells or isolated pituitary slices, respectively (135, 142). However, this concept was recently challenged by several reports showing a different function of endocannabinoids on the HPA axis. In fact, some studies showed that administration of the CB1 receptor antagonist SR141716 in rats is able to induce ACTH and corticosterone release and to produce anxiety-like behavior (143, 144). It is well known that this behavior represents part of the physiological response to stressful stimuli and is, indeed, associated with the hyperactivation of the HPA axis (145). Moreover, compounds able to increase endocannabinoid tone by inhibiting FAAH activity were recently proposed as treatment for anxiety-related disorders because they were shown to reduce restraint-induced corticosterone release (146) and to diminish the anxiety-like response in different tasks (147). In addition, mice lacking CB1 receptor (CB1−/−) are resistant to some actions of anxiolytic drugs (148). In support of the existence of a close interaction between the endocannabinoid system and CRH, it is important to mention that CB1 receptor and CRH mRNAs are coexpressed in PVN neurons, and that CB1−/− mice present increased CRH mRNA levels in this region, indicative of a possible basal alteration of the HPA axis activity due to the disruption of CB1 receptor signaling (58). Therefore, a novel view seems to attribute the endocannabinoid system with a critical inhibitory action on HPA functions. A recent elegant report by Patel et al. (146) shed light on this issue. The authors confirmed previous studies showing that systemic treatment with SR141716 is able to increase serum corticosterone concentrations in basal conditions; more importantly, they found that pretreatment of mice with the same CB1 receptor antagonist before acute restraint stress provokes a potentiation of the restraint-induced rise in serum corticosterone concentrations. In addition, endogenous cannabinoids and, in particular 2-AG, were found to be decreased after a short period of restraint stress, whereas a condition of prolonged stress was associated with an increase in 2-AG concentrations (146). Accordingly, they concluded that endocannabinoid signaling negatively modulates the stress-induced activation of the HPA axis, confirming the notion that a pharmacological increase in endocannabinoid signaling activity may constitute a novel approach to the treatment of anxiety-related disorders (146). These findings reinforce the general concept that the pharmacological administration of cannabinoids may lead to a completely different action when compared with the physiological functions of the endocannabinoid system as shown by experiments using CB1 receptor antagonist or CB1−/− mice.

Besides the hypothalamus, peripheral sites of action, such as pituitary and adrenal glands, could participate in the endocannabinoid modulation of the HPA functions. In cultured human ACTH-producing tumors, WIN 55,212-2 was found to be ineffective in influencing basal ACTH secretion. However, the simultaneous application of WIN 55,212-2 and CRH caused a synergistic action, which was abolished by SR141716, indicating that the activation of CB1 receptor might play a role during CRH-induced activation of ACTH-secreting cells (52). Therefore, in the corticotroph cells, an endocannabinoid tone could interfere with the normal regulation of the adenylate cyclase activity and, thus, with the secretion of ACTH. As mentioned above, a pending question regards CB1 receptor expression and endocannabinoid production at the level of cortical adrenal gland and their putative role in the secretive function of this gland. Further efforts are needed to solve this important issue. Interestingly, our recent unpublished studies indicate that CB1−/− have higher plasma levels of corticosterone but normal levels of ACTH, suggesting a putative regulation of adrenal activity by the endocannabinoid system (our unpublished results).

B. The role of cannabinoids in GH secretion

GH secretion is mainly stimulated by hypothalamic GHRH and by the recently discovered peptide ghrelin (69), whereas somatostatin is the most important negative regulator. Other neurotransmitters such as serotonin, dopamine, and catecholamines can affect GH secretion through modulation of GHRH release. Few data are available concerning the effects of marijuana on GH in humans. Four days of marijuana consumption were shown to inhibit the GH-counteracting response provoked by insulin-induced hypoglycemia (149). Δ9-THC and synthetic cannabinoids were shown to inhibit GH secretion in rodents (150, 151, 152). However, compared with other hormones, it is still questionable whether cannabinoids are able to decrease GH secretion acting exclusively at the hypothalamic level or whether they also directly influence GH pituitary output. Rettori et al. (153) observed that only intracerebroventricular Δ9-THC administration was able to reduce GH secretion, whereas no effect was observed in cultured rat pituitary cells. Interestingly, by incubating fragments of median eminence with Δ9-THC, a significant stimulation of basal somatostatin was found (154); this finding makes it possible to speculate that the inhibitory action of Δ9-THC on GH secretion could be mediated by somatostatinergic activation (154). Recent data point to a functional cross-talk between CB1 receptor and the ghrelinergic system. In fact, hyperphagia associated with intracerebroventricular administration of ghrelin is blocked by pretreating the rats with SR141716 (155). Unfortunately, no data have been provided concerning GH release in this experimental setting. Altogether, these data seem to indicate that the effect of exogenous cannabinoids on GH secretion is located at a suprapituitary level. However, the cannabinoid agonist WIN 55,212-2 inhibited GH secretion in human GH-producing adenomas in culture, and this effect was reversed by the specific CB1 receptor antagonist SR141716, suggesting that cannabinoids are able to directly influence basal GH secretion through CB1 receptor activation, at least in tumoral tissues (52). No data are available on the physiological modulation made by the endocannabinoid system on GH secretion.

C. Cannabinoids and the hypothalamic-pituitary-thyroid axis

Pioneer studies showed that marijuana is able to decrease TSH and thyroid hormones in rats (156, 157) and iodine accumulation in the isolated rat thyroid (158). The lack of changes in TRH secretion in the hypothalamus led the authors to conclude that the cannabinoid effect could be attributed to a direct action at the level of the pituitary or the thyroid gland (157). Recently, Porcella et al. (56) found a CB1 receptor-dependent decrease (30%) in both free T3 and free T4 4 h after the administration of the synthetic cannabinoid agonist WIN 55,212-2 in rats. TSH levels were unaffected, indicating that the thyroid gland itself may be the direct target of cannabinoid action (56). On the other hand, the lack of TSH changes may also be explained by an action of cannabinoids on the levels of thyroid binding protein or on the metabolism of thyroid hormones. More studies are needed to verify these hypotheses. Concerning the physiological roles of the endocannabinoid system, an inhibitory action on TRH neurons through a glucocorticoid-induced inhibition of glutamate transmission was recently proposed (103).

D. The role of cannabinoids in prolactin secretion

There is no general consensus regarding the effect of exogenous cannabinoids on PRL secretion. Early studies in rodents and primates favor an inhibitory role of cannabinoids on PRL release (153, 159, 160, 161, 162) through a CB1 receptor-mediated effect (163). Conversely, some reports showed that cannabinoids may also have either a stimulatory effect (164, 165) or no effect (166) on PRL release. As often occurs in the field of cannabinoids, this controversy may be largely due to the different experimental settings used. The conflicting data may also originate from the biphasic profile of PRL observed after Δ9-THC administration, with an initial increase followed by a marked decrease after time (167). In the same study, the antagonist SR141716 was only able to block the inhibitory effect, whereas no effect was seen toward the cannabinoid stimulatory effect on PRL (167). There is a general agreement that cannabinoid activation of the tuberoinfundibolar dopaminergic neurons controlling PRL secretion is the main mechanism responsible for the inhibition of this pituitary hormone (168, 169). When Δ9-THC was chronically administered to ovariectomized or hypophysectomized female rats or to dispersed pituitary cells in culture, no effect was seen on PRL release, suggesting that the inhibitory cannabinoid effect targets the CNS directly (161). Similar conclusions were drawn from similar models by other authors (153). Recently, exogenous AEA was shown to inhibit PRL release from male rats by acting on the CB1 receptor on dopaminergic neurons located in the medial basal hypothalamus (162). However, like other hormones, it has also been hypothesized that cannabinoids may also affect PRL secretion directly in the pituitary. Indeed, Δ9-THC was able to prevent estrogen-induced PRL secretion in vivo (170) and in vitro (170). The direct effect of cannabinoids at pituitary level was also confirmed by the demonstration that WIN 55,212-2 does not affect basal secretion, but inhibits vasoactive intestinal peptide- and TRH-stimulated PRL release in tumoral pituitary GH4C1 cells (171). WIN 55,212-2 was also able to inhibit PRL secretion in a single case of prolactinoma in culture (52). In conclusion, we can assume that the biphasic action on PRL secretion of exogenous cannabinoids is mediated by an initial activation of CB1 receptor located at the level of the pituitary and followed by a persistent inhibitory action mediated by the activation of the release of dopamine from hypothalamic structures.

E. The role of cannabinoids in modulation of the hypothalamic-pituitary-gonadal axis and fertility

1. In females.

While FSH secretion seems to be unaffected by administration of exogenous or endogenous cannabinoids (172), several pieces of evidence attribute cannabinoids with a strong ability to down-regulate blood LH levels (49, 165, 172, 173). This effect is due to a complete suppression of the secretory pulse of LH (174, 175). In monkeys, chronic administration (18 d) of Δ9-THC was shown to block estrogen and LH surges and the consequent elevation in progesterone (176). However, the same animals developed tolerance to the antireproductive effect of the drug after a few months of treatment (177). In women smoking a single marijuana cigarette with a fixed content of Δ9-THC, a decrease of LH was observed during the luteal phase, whereas no effect was seen on the same hormone in the follicular phase and in the postmenopausal state (178, 179). The sustained use of marijuana (at least four times per week) may cause alterations of the menstrual cycle, such as oligomenorrea; however, no changes were shown in hormonal parameters in a group of 13 pregnant women who continued to smoke marijuana during pregnancy (180). An excess of cannabinoids may also impair regular ovulation, not only acting at the hypothalamic level but also directly affecting ovarian granulosa layers (76).

A general consensus attributes the LH-inhibitory action of cannabinoids to a suprapituitary site of action. In fact, administration of gonadotropins or GnRH was able to induce ovulation or LH release, respectively, even in the presence of high levels of Δ9-THC (174, 175). However, a report showed that cannabinoids are not able to block the basal GnRH secretion from hypothalami in vitro (165). This last finding suggests that cannabinoids indirectly modify GnRH secretion by negatively modulating the activity of neurotransmitters known to facilitate GnRH secretion, such as norepinephrine (165) and glutamate (181), and by stimulating those modulators known to down-regulate GnRH secretion, such as dopamine (182), GABA (183), opioids (184), and CRH (185). The stimulatory effect of cannabinoids on dopaminergic neurons is well known (186), however their impact on the brain dopaminergic activity varies as a function of the gonadal status, as demonstrated by several lines of evidence (187). In particular, it has been shown that steroid hormone receptors mediate the well known Δ9-THC-facilitation on sexual behavior (188) exerted, as recently shown, by CB1 receptor activation (189). Moreover, in the same study Mani et al. (189) reported that an interaction between progesterone and dopamine receptor type 1 (D1) is required for Δ9-THC-facilitated sexual receptivity in female rats.

However, although pharmacological studies have helped to explain the relevant role of the cannabinoids in modulation of the hypothalamus-pituitary-gonadal axis and sexual behavior, it is not yet known how, where, and under what circumstances the endocannabinoids are produced to do so. The recent findings of fluctuation during the ovarian cycle of AEA in both hypothalamus and pituitary (49) allowed some authors to speculate that endocannabinoids may influence hormonal secretion and sexual behavior by directly targeting the CB1 receptor (190). Furthermore, an important production of endocannabinoids was found in the ovary, in particular at the time of ovulation, making it possible to hypothesize that the endocannabinoids may help to regulate follicular maturation and development of the ovary (74).

The uterus contains the highest level of AEA detected so far in mammalian tissues, and it is the only tissue where AEA is the main component (up to 95%) of N-acylethanolamides (191). This observation, together with the expression of CB1 receptors in preimplantation embryos (80), recently prompted strong efforts focused on the role of the endocannabinoid system during early pregnancy and in the modulation of embryo-uterine interactions. High levels of AEA adversely affect embryo development and implantation through CB1 receptor activation (192), whereas low levels of AEA promote embryonic growth and differentiation (193, 194, 195). It is therefore evident that the degradation of AEA by FAAH is a crucial enzymatic checkpoint in the control of reproduction. Notably, a strong inverse correlation was described between levels of FAAH activity in maternal peripheral blood mononuclear cells and spontaneous miscarriage in women (196). In addition, FAAH activity is lower, and consequently AEA higher, in patients who fail to achieve pregnancy during in vitro fertilization embryo transfer in comparison to patients who become pregnant (197). Furthermore, AEA levels in the mouse uterus are inversely related to uterine receptivity for implantation, being higher with uterine refractoriness to blastocyst implantation (191, 198, 199) and lower at implantation sites (194). We can therefore conclude that high levels of maternal AEA are detrimental to early placental and fetal development. In favor of this hypothesis, it was recently shown that high levels of FAAH are present in the cytotrophoblast, presumably to prevent the transfer of AEA from maternal blood to the embryo (200). A series of studies by Maccarrone et al. (72, 195) showed that the activity of FAAH is under the strict regulation of several hormones, such as progesterone, leptin, and FSH, very well-known modulators of fertility. Importantly, by using genetic or pharmacological blockade of the CB1 receptor, it was very recently demonstrated that an impairment in endocannabinoid signaling leads to a retention of a large number of embryos in the mouse oviduct, leading to pregnancy failure. This is due to a profound impairment of a coordinated oviductal smooth muscle contraction and relaxation (79). The authors propose that their findings may have strong implications for ectopic pregnancy in women because one major cause of tubal pregnancy is embryo retention in the fallopian tube (79). Consistently, both endogenous and exogenous cannabinoids exert a CB1 receptor-mediated relaxant effect, not only on the oviductal smooth muscle but also on the human pregnant myometrium, highlighting a possible role of endocannabinoids during human parturition and pregnancy (78). In fact, pregnancy also seems to be tightly controlled by the endocannabinoid system (200). In summary, all the steps starting with fertilization up to pregnancy seem to be tightly modulated by endocannabinoids, reinforcing the concept that the endocannabinoid system should be considered not only as a central neuromodulator but also as a physiological actor in a wider scenario.

2. In males.

Cannabinoids also were shown to decrease LH in males (201, 202). Although there is still no general consensus, chronic cannabinoid use in several species seems to decrease testosterone production (203) and secretion (201, 202), to suppress spermatogenesis, and to reduce the weight of testes and accessory reproductive organs (204). The important effects of cannabinoids on the gonadal system are mainly attributed to CB1 receptor activation, as demonstrated by using specific CB1 receptor agonists and antagonists (151, 205). Definitive confirmation was provided by a recent study showing that AEA injected ip is able to lower LH and testosterone in wild-type mice but not in CB1−/− mice (71). Interestingly, the testis is known to express CB1 receptor (70) and to synthesize endocannabinoids (206). The cannabinoid effect in down-regulating testosterone circulating levels may explain the reduced copulatory behavior in male rodents exposed to Δ9-THC (207).

The finding that male genital tract fluids contain significant concentrations of endocannabinoids (74) suggests that these lipid-signaling molecules may influence important processes controlling sperm/egg functions and gamete interactions. Studies with sea urchin gametes provided the first evidence that cannabinoids, in particular AEA, are able to directly inhibit achrosome reaction and sperm fertilization capacity (208). On the other hand, seminal plasma contains high amount of AEA, and this may contribute to maintaining sperms in a quiescent metabolic condition (74). The content of AEA decreases progressively in the uterus, oviduct, and follicular fluid, and this change in endocannabinoids may render sperms suitable for capacitation and fertilizing ability (74, 209). Furthermore, as shown in sea urchin, the eggs may have the capacity to release AEA after activation by the fertilizing sperm (210), inducing a CB1 receptor activation that might be able to prevent polyspermic fertilization by blocking the acrosome reaction in other sperm (209).

In humans, CB1 receptor activation by AEA was also shown to reduce sperm mobility by affecting mitochondrial activity, and to inhibit capacitation-induced acrosome reaction. Importantly, these effects are inhibited by the CB1 receptor antagonist SR141716 (75). It is therefore reasonable to hypothesize that AEA levels might be increased in different pathological conditions of the male reproductive tract. In these cases, the pharmacological blockade of the endocannabinoid system might be helpful in the treatment of some forms of male infertility (75).

In conclusion, it appears that the endocannabinoid system plays an important role in the regulation of the hypothalamus-pituitary-gonadal axis both in females and in males, and fertility may be affected by cannabinoid drugs. This evidence may represent an important issue in clinical endocrinological praxis. In the light of the widespread use of marijuana as a recreational drug among young people, subtle alterations of the gonadal hormonal profile or in fertility may therefore be attributed to a concomitant use of cannabis derivatives. On the other hand, the results of human epidemiological studies have not always been clear in confirming this negative impact (211), and more detailed research on this topic is needed in the future before drawing definitive conclusions.

IV. Endocannabinoid System in the Modulation of Energy Balance

Section:

Two notions highlight the importance of the endocannabinoid system in the regulation of food intake and energy metabolism. The first is the finding

of a high degree of evolutionary conservation of the role of this system in the regulation of feeding responses (212). The second is the observation that high levels of endocannabinoids in maternal milk are critically important for the initiation of the suckling response in newborns (213).

A. Animal studies before the discovery of endocannabinoids

Animal models are ideal tools for elucidating the putative mechanism(s) of cannabinoids in the control of energy metabolism. The studies performed in different species to test the orexigenic properties of Δ9-THC up to the discovery of endocannabinoids are summarized in Table 2 (214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244). From a general point of view, one can say that rather contradictory results were obtained in these experiments. The ambiguous data could likely be attributed to differences in the animal model and in the experimental procedures used. Moreover, in early studies using marijuana extracts, comparisons between various experimental data sets are extremely difficult due to the variability of the activity of cannabis derivatives, the dosages, and the routes of administration. In general, early studies using low doses of cannabinoids showed a reliable increase in food intake. When doses of Δ9-THC above 10 mg/kg were used, a concomitant decrease in food intake was observed due to the confounding factors given by the sedative effect of the drug. Studies employing high amounts of Δ9-THC should thus be viewed with caution in terms of effects on appetite and body weight. This is also the reason why, in reviewing the studies published between 1965 and 1975, Abel reported an increased food intake after cannabinoid administration only in 3 of 25 experiments (245). In 1998, Williams et al. (246) provided a very convincing and well-performed experiment to characterize the orexigenic property of Δ9-THC. The authors maximized the ability to detect hyperphagia by adopting a prefed paradigm in which the animals were characterized by low baseline food intake before drug administration. In this experimental setting, Δ9-THC was given orally at increasing dosage before unrestricted access to a standard diet. The authors observed that the maximum effect of the drug (1.0 mg/kg) was far greater than previously reported results, showing a 4-fold increase in food consumption over 1 h. Importantly, this hyperphagic effect was largely attenuated by pretreatment with the CB1 receptor antagonist SR141716, strongly supporting the notion that CB1 receptor activation mediates the hyperphagic effect of Δ9-THC (247). In this experiment, it was also reported that at doses of Δ9-THC higher than 1.0 mg/kg, the rats become unable to overeat due to the presence of motoric and sedative side effects (246). These results strongly suggest that the anorectic effect of Δ9-THC shown by many previous reports was indirectly due to the sedated state induced by high doses of the drug.

B. Studies in humans with exogenous cannabinoids before the discovery of endocannabinoids

Abel (245) also critically reviewed the studies aimed at proving the stimulating effect of cannabis on hunger in humans. However, the lack of scientific thoroughness of these earlier studies led Abel to conclude that the putative cannabis-induced hunger effect was still far from being proven (245). Greenberg et al. (248) were the first to systematically assess, under rigorous experimental conditions, the effect of a well-defined amount of Δ9-THC in terms of changes in feeding behavior and in body weight in humans. Both parameters increased after the first few days of the experiment. However, after this period, body weight continued to rise, averaging 2.3 kg across the whole 21-d period study, whereas a stabilization of energy intake was observed. This pioneer study already suggested that the ability of cannabinoids to stimulate hunger may vanish with time, whereas a possible metabolic effect of the drug may remain active longer (248). Nonetheless, later studies did not investigate the metabolic idea further, preferring to concentrate interest on the ability of cannabis to stimulate hyperphagia and overconsumption of highly palatable food at the central level. In 1986, Foltin et al. (249) noted a relevant increase in frequency and consumption of snack foods induced by marijuana only in the periods of social facilitation and environmental familiarity and not when the subjects were alone, indicating on the one hand a strong link between recreational use of the drug and its orexigenic properties and, on the other hand, the ability of marijuana to drive the tendency for palatable food. This hypothesis was further substantiated by the same group a few years later when increased total food intake particularly related to consumption of palatable food (sweet solid snacks) was observed as a main effect of smoked marijuana (250).

The stimulating effect of cannabinoids on appetite observed in healthy subjects promoted assessment of the efficacy of a cannabinoid treatment for clinical syndromes featuring loss of appetite or weight, such as cancer or AIDS-associated anorexia (251, 252, 253), or as adjuvant therapy to limit nausea and vomiting symptoms associated with most chemotherapeutic drugs (254). In 1985, the U.S. Food and Drug Administration officially approved the use of Δ9-THC (commercially named Dronabinol) for the treatment of chemotherapy-induced nausea and vomiting refractory to other drugs. In 1992, Dronabinol was approved for the treatment of patients with HIV-induced wasting syndrome. Recently, Dronabinol was also proposed as an orexigenic drug in patients suffering from Alzheimer's disease (255).

The most comprehensive data are those obtained when Dronabinol was administered in HIV patients with wasting syndrome (252, 256, 257, 258, 259). To varying degrees, the drug was able to mildly increase appetite and energy intake in all studies. However, a marked improvement in mood was also documented, raising the question of whether the positive effect in energy balance may derive from a specific action of cannabinoids in the brain areas controlling food intake or may be simply due to a generalized change in the sense of well-being. Intriguingly, in some reports, a significant gain was found in body fat mass associated with minimal changes in appetite rating and food intake (255, 258). At that time, this finding remained unexplained. However, with the current knowledge of CB1 receptor expression at the level of the adipose tissue (58, 59), we can hypothesize that the increase in fat mass of HIV patients was probably due to a direct lipogenic action of Δ9-THC. In this context, it is still unknown, and it would be of great relevance to investigate whether the administration of Dronabinol can improve the pathological changes in fat distribution induced by the concomitant retroviral therapy in patients with AIDS (260).

C. Endocannabinoid functions at mesolimbic level to regulate rewarding properties of food

After the finding of the hyperphagic effect of Δ9-THC mediated by CB1 receptor activation, Williams and Kirkham (261) reported that endocannabinoids were also able to stimulate hunger in a dose-dependent manner. The degree of overeating induced by 1 mg/kg AEA was only a 2-fold increase over a 3-h test, therefore less than that obtained with the same dosage of Δ9-THC. However, Δ9-THC-induced hyperphagia was restricted to the first hour of testing, whereas the AEA effect was evident later when the inhibitory effects of the prefeed started to wane (261). The authors speculated that administration of AEA may represent an amplification of endocannabinoid activity associated with the normal, episodic pattern of meal-taking in rats (261).

Importantly, the effect of AEA was completely blocked by pretreating the animals with SR141716, confirming the pivotal role of CB1 receptor activation in the hyperphagic effects of endocannabinoids (247, 262). Similar conclusions were derived from other studies in which AEA was able to exert an appetite-stimulating effect even at very low doses in mice (0.001 mg/kg) (263) and 2-AG was capable of promoting feeding behavior (264). These data therefore make it possible to attribute the endocannabinoid system with an important role in the processes underlying the motivation to obtain food. It is suggested that endocannabinoids gradually increase during intermeal intervals, reaching a critical level where motivation to eat is triggered. Accordingly, the longer the time since the last meal, the greater the activity in relevant endocannabinoid circuits, and consequently the higher the motivation to eat (265). The findings of increased levels of AEA and 2-AG in the fasting condition in the nucleus accumbens and a decline of 2-AG concomitant with the feeding state strongly support this hypothesis (264). Interestingly, unchanged levels of endocannabinoids were shown in the cerebellum, a region not involved in the control of feeding, further confirming the notion that endocannabinoids are produced in situ and on demand (264).

With the advent of CB1 receptor-specific antagonists (Table 3), it became clear that, even when injected alone, these compounds are able to modify ingestive behavior. An ip injection of SR141716 was found to significantly reduce sucrose or alcohol intake and craving in rodents (266, 267, 268) and in marmosets (269), leading to the hypothesis that the activation of the endocannabinoid system may alter the appetitive value of ingested substances. This idea is consistent with the evidence in favor of a facilitatory function of the endocannabinoid system on brain reward circuits (266, 269). Evidence therefore suggests that endocannabinoids bring forward the onset of eating in satiated animals and increase the incentive value of the food regardless of the quality of the macronutrients ("incentive hypothesis") (270). Other findings, however, resembling the "marshmallow effect" in marijuana smokers (245), have been interpreted in terms of an endocannabinoid action toward a preference to eat highly palatable food ("orosensory reward hypothesis") (271). In favor of this latter hypothesis, there are several reports indicating the ability of CB1 receptor blockade to decrease the rewarding properties of addictive drugs (186, 272, 273, 274). It is now clear that the endocannabinoid system participates in the modulation of "reward/reinforcement" circuitries and its manipulation is able to influence reward-related behaviors (275). The high expression of CB1 receptor in areas involved in reward constitutes a strong indication that the endocannabinoid system is directly involved in various physiological functions

controlled in these brain regions, including feeding (43). The reward/reinforcement circuitry of the mammalian brain consists of a series of synaptically interconnected brain nuclei associated with the medial forebrain bundle, linking the VTA, the nucleus accumbens, and the ventral pallidum (275). This circuit is implicated in the pleasure produced by natural rewards, such as food, addictive drugs, and sex, and it is the neural substrate of drug addiction and addiction-related phenomena, such as craving and dysphoria induced by withdrawal (275). In such a framework, food intake acts on dopamine, opioid, serotonin, and noradrenaline neuronal fibers, which connect the hindbrain and midbrain to the hypothalamus to modulate the action of feeding and satiety factors (276).

The most relevant reward pathway is represented by the mesolimbic dopaminergic system. It has been shown that increased levels of extracellular dopamine and its metabolites are found within the nucleus accumbens after ingestion of highly palatable food (277). Moreover, administration of a dopamine D1 agonist reduces food intake (278). Both CB1 receptor and endocannabinoids were found in the rat limbic forebrain (279), in which colocalization with dopamine D1 and D2 and CB1 receptor were described (280). Psychoactive drugs such as marijuana, ethanol, and also pleasant stimuli or palatable food are known to induce the release of dopamine in specific brain regions (281). A correlation between limbic endocannabinoid/dopamine levels and craving for tasty food is thus presumed to occur (275). Verty et al. (282) recently substantiated the hypothesis of the existence of cannabinoid-dopamine interactions in feeding behavior, demonstrating that the dopamine D1 antagonist SCH 23390 attenuated feeding induced by Δ9-THC. The endocannabinoid system also provides retrograde control of synaptic transmission onto the VTA dopaminergic neurons, where the postsynaptic synthesis of endocannabinoids is under the control of somatodendritically released dopamine (108).

A relevant interplay also exists between the endocannabinoid system and the endogenous opioid peptides (283). Both systems are linked to central reward processes, and there is increasing evidence supporting an important functional cross-talk between the two systems, in relation to a wide range of physiological processes, including appetite. Several reports indicate that opioid receptor agonists increase food intake (284, 285, 286), whereas opioid antagonists induce anorectic effects (287). Gallate and McGregor (267) found that the facilitatory effects of a cannabinoid agonist on responding to palatable solutions were reversed not only by CB1 receptor antagonism but also by naloxone, an opioid receptor antagonist. The existence of cross-talk between the endocannabinoid and opioid systems in controlling food intake was also confirmed by several studies in which naloxone and SR141716 synergistically depress food intake at doses that do not alter food intake on their own (287, 288). However, a recent finding seems to localize the interaction between opioids and endocannabinoids involved in feeding behavior not at the mesolimbic system level but, preferentially, at the level of the PVN of the hypothalamus. In fact, SR141716 was able to attenuate morphine-induced feeding only when the opioid was directly injected in the PVN and not in the nucleus accumbens. According to this last finding, the endocannabinoid system appears to participate in the opioid-mediated enhancement of rewarding properties of food in the hypothalamus and not in the nucleus accumbens (286).

According to the involvement of serotonin in the control of feeding behavior (289), the interaction of the endocannabinoid system with the serotoninergic system has also been investigated. However, the administration of a CB1 receptor antagonist in rats combined with dexfenfluramine, an anorectic drug stimulating the release of serotonin, led to additional but not synergistic effects on reducing food intake, which is consistent with the hypothesis that the two pathways work via independent mechanisms of action (288). This notion is important, because it makes it possible to exclude a synergistic effect in a possible future combination of antiobesity drugs such as those inhibiting serotonin reuptake, like sibutramine (290) and CB1 receptor antagonists.

D. The endocannabinoid system as a new hypothalamic player in the regulation of food intake

A complex and redundant neuronal hypothalamic network provides high levels of adaptability of feeding behavior to various central and peripheral stimuli (291). Redundancy in appetite-stimulating signaling is conceivable in view of the vital importance of feeding for survival (291). Whereas defects in anorexigenic signaling pathways almost always lead to obesity, loss of orexigenic signals rarely results in a lean phenotype. An example of this redundancy in orexigenic hypothalamic signaling systems is provided by mice lacking neuropeptide Y (one of the most important appetite-stimulating neuropeptides) where compensatory mechanisms are likely to be activated (292). Signals coming from various peripheral organs, such as the liver, gastrointestinal tract, and adipose tissue, are conveyed mainly at the hypothalamic level to constantly inform the brain about the state of nutrition (291, 293). An example of such peripheral control is the adipocyte-derived hormone leptin, which acts on receptors located in the hypothalamus (291). A milestone in the identification of the endocannabinoid system as a new player in the regulation of food intake at hypothalamic level was the finding that leptin is a strong modulator of hypothalamic endocannabinoid levels (294). Di Marzo et al. showed that acute leptin treatment reduced AEA and 2-AG not only in the hypothalami of normal mice but also in mice lacking leptin signaling. They also described the defect in leptin signaling as being constitutively associated with elevated hypothalamic levels of endocannabinoids. In these animals, SR141716 was able to reduce food intake, confirming the anorectic properties of the compound (294). These findings suggest that, at least in genetically modified animal models, obesity is associated with a chronic hypothalamic overactivation of the endocannabinoid system, which may in turn explain the hyperphagic behavior of the animals having leptin signal impairment. However, before giving a general value to this assumption, the intrahypothalamic amount of endocannabinoid levels during the development of obesity in normal rodents eating a high-fat diet must be investigated. Nevertheless, endocannabinoids are variably produced in the hypothalamus of normal animals. In fact, 2-AG levels increase during acute fasting, decline as the animals are refed, and return to normal values in satiated animals (264, 295). However, a long period of diet restriction (12 d) was found to be associated with reduced levels of 2-AG in the hypothalamus (295). The authors interpreted these data observing that the decrease of 2-AG levels in mice after a prolonged diet may represent a general psychobehavioral strategy for intermittent starvation when food is scarce (295).

As mentioned above, the hypothalamus is not the cerebral area where the highest levels of CB1 receptor expression are found (24, 36, 38). However, studies using [35S]GTPγS binding indicated that the hypothalamic CB1 receptor coupling to G proteins is more efficient than in other cerebral areas known to be a site of high CB1 receptor expression, such as the hippocampus or the entopeduncular nucleus (43). On the other hand, it is also evident that CB1 receptors are present at a very high density in the brain compared with other receptors. Therefore, even regions with a relatively lower density of CB1 receptors, such as the hypothalamus, contain a significant number of receptors. Both these factors thus probably explain the ability of hypothalamic CB1 receptors to strongly affect the functions of this brain region. Interestingly, no changes in CB1 receptor expression have been shown at the level of hypothalamus after diet modification (296). The direct involvement of the hypothalamus in the modulation of food intake operated by endocannabinoids was also demonstrated by the significant hyperphagic effects of AEA directly administered into the ventromedial nucleus and by the inhibition of this effect obtained by the injection of SR141716 via the same route (297).

It was only during the last few years that the interaction of CB1 receptor and endocannabinoids in feeding-regulating pathways started to be elucidated in detail. The CB1 receptor is expressed in key hypothalamic peptidergic systems, such as those producing CRH in the PVN, cocaine-amphetamine-related transcript in the dorsomedial nucleus, and melanin-concentrating hormone and orexins in the lateral hypothalamus-perifornical area (58). Importantly, these data were recently confirmed by the demonstration that CB1 receptor activation strongly augments the orexin-A-stimulated intracellular pathway (88). CB1−/− mice also possess increased CRH and reduced cocaine-amphetamine-related transcript expression, indicating that the genetic impairment of the endocannabinoid system may affect the pattern of gene expression of peptides involved in the regulation of food intake (58). Conversely, the neuropeptide Y/agouti-related protein system in the arcuate nucleus does not seem to be directly targeted by endocannabinoid action (58, 294). This fact confirms that orexigenic pathways are less critical (or at least functionally more redundant) in the chronic maintenance of energy balance (298). Functional cross-talk between CB1 receptor and melanocortin receptor type 4 (MCR4) has been recently highlighted by the finding of the synergistic action of subanorectic doses of SR141716 and of a MCR4 agonist administered together (299). Furthermore, the same authors showed that the orexigenic impulse given by the administration of CB1 receptor agonists is not blocked by the costimulation with MCR4 agonists, whereas CB1 receptor antagonists are able to inhibit the stimulation of food intake induced by MCR4 antagonists. Consequently, the authors hypothesized that the melanocortin receptor signaling in the hypothalamic regulation of food intake is upstream of the activation of the endocannabinoid system (299).

The mechanism(s) of action of the endocannabinoids at hypothalamic synaptic level are still a matter of debate. Great progress has recently been made by the finding that postsynaptically released endocannabinoids acting at presynaptic CB1 receptors are able to decrease glutaminergic transmission onto CRH-producing neurons, resulting in an inhibition of CRH release (103). This release of endocannabinoids from the parvocellular neurons is stimulated by a nongenomic effect of glucocorticoids. Therefore, it is conceivable that the well-known regulation of food intake by

glucocorticoids may partly derive from functional cross-talk with the endocannabinoid system (300). The same inhibitory mechanism mediated by glucocorticoids through an activation of the endocannabinoid system has also been proposed for other hormones and neuropeptides such as oxytocin and vasopressin (103). In this sense, we may speculate that the recently described interaction between endocannabinoid and the oxytocin system in modulating food intake (301) may derive from the same fast feedback mechanism mediated by nongenomic glucocorticoid inhibition.

Despite the dogma that neurons do not utilize fatty acids for energy, a growing body of evidence points to a critical role for both fatty acid production and utilization in regulating hypthalamic neurons that regulate food intake (302). In fact, inhibitors of fatty acid synthase are capable of greatly affecting appetite in an anorexigenic manner (303, 304). In such a scenario, it has recently been proposed that via CB1 receptors, endocannabinoids may modulate the fatty acid synthetic pathway in the hypothalamus, and the inhibition of the hypothalamic expression by rimonabant may explain the anorexigenic properties of cannabinoid antagonists (62).

E. The peripheral effect of the endocannabinoid system in the modulation of metabolic functions

Several lines of evidence are currently converging, indicating that the effects of CB1 receptor blockade on food intake and body weight are not limited to a central mode of action. An early report describing the effect of CB1 receptor blockade on changes in food intake and in body weight was, in this sense, highly predictive of a mechanism of action not limited to the mesolimibic or hypothalamic circuits. In fact, Colombo et al. (305) were the first to demonstrate, in lean rats fed with a standard diet, that the tolerance to the anorectic effects of two different doses of SR141716 (2.5 and 10 mg/kg) develops rather rapidly (5 d). Nevertheless, the body weight loss in SR141716-treated rats persisted for 14 d, well beyond the drug effect on food intake. At that time, the authors were not able to explain this body weight loss that was not related to a decrease in food intake, and they merely hypothesized a stimulatory action of SR141716 on the energy expenditure (305). However, in the last 2 yr, the use of CB1−/− mice has represented an important tool to substantiate further the hypothesis of an additional effect of endocannabinoids in peripheral organs. Indeed, the lack of CB1 receptor in mutant mice causes hypophagia and body fat reduction. Importantly, pair-feeding experiments showed that in young CB1−/− mice, the lean phenotype is predominantly caused by decreased caloric intake, whereas in adult CB1−/− mice metabolic factors appear to be the major cause of the lean phenotype. These experiments therefore suggested that the endocannabinoid system might regulate central food intake-related mechanisms at young ages, but that this function diminishes with age (58). These observations converge on the idea that additional peripheral food intake-independent metabolic functions may participate, or even predominate, in the control of energy balance exerted by the endocannabinoid system (58).

Even more prominent differences in terms of body weight regulation are obtained when a high-fat diet is administered to adult CB1−/− mice and wild-type littermates. In contrast to wild-type littermates, CB1−/− mice do not display hyperphagia or reduction of their relative energy intake and were resistant to diet-induced obesity (DIO) (306). Importantly, the obesity-prone diet induced a significant increase of fasting glycemia in the two genotypes, but the sensitivity to insulin remained unchanged in CB1−/− mice, whereas it was significantly reduced in the wild-type animals (306).

The expression of CB1 receptor in adipocytes and the ability of SR141716 to block lipogenesis stimulated by cannabinoids represent a first important step forward in understanding the peripheral mechanisms of action of the endocannabinoid system in regulating metabolic processes (58). Moreover, the presence of CB1 receptor is increased in mature adipocytes compared with preadipocytes (59, 60), indicating that CB1 receptor activation is likely needed more for metabolic processes than for differentiation. Importantly, a recent study shed further light on the mechanisms of action of the endocannabinoid system on adipose tissue. By using SR141716 in DIO mice, Jbilo et al. (307) were able to reverse the phenotype of obese adipocytes at both macroscopic and genomic levels. They showed that a major restoration of white adipocyte morphology similar to lean animals occurred in adipocytes derived from obese animals after CB1 antagonist treatment. More importantly, they found that the major alterations in gene expression levels induced by obesity in white adipose tissue were mostly reversed in SR141716-treated obese mice. Importantly, the transcriptional patterns of treated obese mice were similar to those obtained in the CB1−/− mice fed with a high-fat diet, supporting a CB1 receptor-mediated process. Functional analysis of these modulations indicated that the reduction of adipose mass by the drug was due to enhanced lipolysis through the induction of enzymes of the β-oxidation and tricarboxylic acid cycle; increased energy expenditure, mainly through futile cycling (calcium and substrate); and a tight regulation of glucose homeostasis. In particular, in this last context the SR141716-induced increased expression of glucose transporter 4, the insulin-responsive glucose transporter, appears very important (307). This finding makes it possible to hypothesize that cannabinoid antagonists may also be attractive drugs in fighting diabetes. Altogether, these data confirmed that the endocannabinoid system has a major role in the regulation of energy metabolism in adipocytes. Importantly, CB1 receptor expression has been found to be higher in adipocytes derived from obese animals compared with lean controls (59). Similar to the finding of higher levels of endocannabinoids in the hypothalamus derived from obese animals, the overexpression of CB1 receptor in adipocytes of obese rats seems to confirm the notion that hyperactivity of the endocannabinoid system is associated with the obesity state. However, this up-regulation of CB1 receptor expression in fat pads derived from rodents has not been confirmed in adipocytes derived from sc fat of obese women (60); on the other hand, a partial limitation of this study is that CB1 receptors have not been measured in visceral fat tissue that is supposed to be more prone to the endocannabinoid action. Finally, the increase in levels of adiponectin in Zucker obese rats chronically treated with SR141716 in vivo (59) and in 3T3 F442A adipocytes acutely stimulated with the CB1 receptor antagonist in vitro (59) points to a close relationship between CB1 receptor blockade and the production of this antiatherogenic and antidiabetic adipocyte-derived protein (308). The quick and strong improvement of hyperinsulinemia detected after a very short-term treatment with SR141716 (4 d) in obese Zucker rats was also attributed to an increase in adiponectin (59). However, the well-known reduction in food intake and the consequent body weight loss displayed at the beginning of SR141716 treatment may be the most obvious explanation for the changes in adiponectin levels. The ability of long-term treatment with SR141716 to enhance the circulating levels of adiponectin was further confirmed in DIO mice (309).

In the last few years, several studies using different CB1 receptor antagonists confirmed the hypothesis that a potential peripheral mode of action of pharmacological CB1 receptor blockade may play a relevant role in the final weight loss effect. Ravinet-Trillou et al. (310) found that long-term (40 d) treatment with two different dosages of SR141716 (3 and 10 mg/kg, respectively) produces a marked acute hypophagia in DIO mice only in the first few days of treatment, followed by the development of tolerance to the anorectic effect of the drug. However, the effect on body weight was sustained until the end of the 5-wk experiment compared with DIO mice treated with the vehicle. The significant difference in weight of white adipose pads between SR141716- and vehicle-treated animals confirmed that weight loss was accompanied by a decrease in adipose tissue. Similar data showed a rapid tolerance to the anorectic action despite a sustained and prolonged effect on body fat loss also being obtained when obese Zucker rats were treated for 14 d with SR141716 (59). Importantly, another CB1 receptor antagonist, AM-251, produced similar effects in DIO mice (311).

Very recently, Poirier et al. (309) monitored weight and metabolic marker changes in three groups of mice after establishing a condition of obesity by a 5-month high-fat diet. Two groups of animals were maintained on a high-fat diet, but one was treated for 10-wk with 10 mg/kg SR141716 and the other one with a vehicle. A third group received a dietary switch to standard food after the 5 months on a high-fat diet. SR141716 induced a weight loss of approximately of 78% in comparison to the weight of the animals receiving the vehicle. More importantly, the antiobesity effect of the drug was equivalent (both in terms of time course and maximum effect) to that achieved by switching obese mice to a normal diet (309). Again, the authors demonstrated that the anorectic effect of the CB1 receptor antagonist vanished with time because the energy intake in the SR141716-treated animals was equivalent to animals on a high-fat diet during the last 6 wk of the experiment and significantly greater than in the group receiving standard diet. Consistent with a previous report (310), the SR141716-induced weight loss was accompanied by normalization of leptin, insulin, and glucose levels (309). Notably, SR141716 also normalized triglycerides and low-density lipoprotein-cholesterol. Moreover, the high-density lipoprotein (HDL)-cholesterol/low-density lipoprotein-cholesterol ratio after SR141716 treatment was significantly higher than in the other two groups (309). Whether this effect on lipid metabolism is indirectly related to an elevation of adiponectin is still a matter of debate.

Shearman et al. (312) recently showed that a 9-d treatment of DIO mice with the CB1 receptor antagonist AM251 increases uncoupling protein (UCP)-1 and UCP-3 mRNA expression level in brown adipose tissue, suggesting that CB1 receptor blockade may contribute to increased thermogenesis. Moreover, Liu et al. (61) found that a 7-d treatment with SR141716 induces an increase in basal oxygen consumption compared with the vehicle in ob/ob mice. The authors were not able to identify the mechanism by which SR141716 treatment is able to affect energy expenditure. A start on clarifying the molecular mechanism by which treatment with SR141716 may favor thermogenesis has been made with the microarray experiment performed by Jbilo et al. (307). These data suggest that the cannabinoid antagonist treatment is able to stimulate the expression of genes favoring energy dissipation through mitochondrial heat production in brown adipose tissue (307). However, it should be mentioned that in vivo

microdialysis studies showed that SR141716 increases noradrenaline outflow in rat anterior hypothalamus, suggesting a possible central stimulation of efferent sympathetic activity (313). Importantly, Liu et al. (61) also showed that a 7-d treatment of SR141716 induces a significant increase in glucose uptake in isolated soleus muscle. This activity might contribute to the improved hyperglycemia seen after SR141716 treatment in previous studies. As shown in Fig. 2, we found that the soleus muscle derived from obese mice contains increased levels of CB1 receptor compared with lean controls, further confirming the hypothesis of a hyperactivity of the endocannabinoid system associated with a condition of obesity.

Hepatocytes, key players in the metabolic processes, were not considered as a target of endocannabinoid action for a long period of time. However, substantial amounts of 2-AG are present in the liver (1.15 nmol/g tissue), and this quantity is nearly double the amount detected in other peripheral organs (295). These observations suggested the idea that the liver might be a new target of endocannabinoid action. Very recently, Osei-Hyiaman et al. (62) strongly substantiated this hypothesis by a series of experiments in which they identified the liver as a primary site for endocannabinoid-mediated modulation of lipogenesis. In fact, probably via inhibition of adenylate cyclase, the cannabinoid agonist HU210 stimulates the expression of several genes involved in the de novo synthesis of fatty acids, such as lipogenic transcription factor SREBP-1c and its targets acetyl-CoA carboxylase-1 and fatty acid synthase. The inhibition of this lipogenic response by SR141716 and its absence in CB1$^{-/-}$ mice confirms the lipogenic role of CB1 receptors localized in hepatocytes. However, more importantly, the authors found that the marked increase in the basal rate of hepatic fatty acid synthesis as well as the development of hepatic steatosis observed after the administration of high-fat diet were blunted by SR141716 and absent in CB1 receptor knockout mice. High-fat diet also induces an increase in the number of CB1 receptors and in hepatic levels of AEA, strongly suggesting that the blockade of the endocannabinoid system plays an important protection against the pathological consequences of a fat diet in the liver (62). These data pave the way to hypothesize the clinical use of CB1 antagonists in preventing or reversing the development of fatty liver.

Another recent report showed that cannabinoids inhibit AMP-activated protein kinase activity in the liver (314). A decrease of AMP-activated protein kinase activity is known to lead to increased storage of energy, particularly in the form of fat, in hepatocytes. This mechanism may contribute to explaining the role of endocannabinoids in promoting the development of hepatic steatosis. Based on the whole body result of these data, it has been hypothesized recently that the hepatic endocannabinoid system may represent a target for the treatment of nonalcoholic fatty liver disease (315).

A considerable amount of evidence suggests that the endocannabinoid system may regulate food intake by also acting in the gastrointestinal tract. Importantly, the concentration of AEA in intestinal tissue increases during food deprivation in rats, reaching levels that are 3-fold greater than those needed to halve maximally activated CB1 receptor and 7-fold higher than the amount detected after refeeding. This surge in AEA levels may, together with the increase in the CNS, be another hunger signal to promote feeding (316). In general, we can conclude that through multiple interactions, endocannabinoids may modulate food intake also at the level of the gastrointestinal tract.

F. Oleoylethanolamide: a new anorectic fatty acid amide

Another endogenous lipid, a monounsaturated fatty acid ethanolamide, named oleoylethanolamide (OEA), was recently proposed as an important modulator of food intake (317). OEA is an analog of AEA, but the activation of any of the known cannabinoid receptors cannot explain its pharmacological effects. Recently, Piomelli's group elucidated that its action is through an activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α (318).

Peripheral administration of OEA causes a potent and persistent decrease in food intake, but this compound is completely ineffective when administered centrally (316, 317, 318). OEA-induced anorexia is not caused by nonspecific behavioral effects, because no aversion or illnesses have been reported after the peripheral administration of the compound (319). Interestingly, similar to the effects described after the administration of capsaicin (vanilloid type 1 receptor agonist) and of the PPAR-α agonist Wy-14643, a short-term reduction in heat expenditure and locomotor activity has been observed after the peripheral administration of OEA (319). However, the mechanisms underlying the reduction in motor activity remain unclear (319). OEA not only acts as a satiety signal, but also reduces body weight gain and serum lipid levels in genetically obese rats and in DIO mice (318). Through the direct activation of PPAR-α, OEA may stimulate lipolysis and fatty acid oxidation (320). However, when administered orally (321), its tissue distribution is mainly at gastrointestinal levels rather than in other visceral organs controlling metabolism, supporting the hypothesis that OEA acts on PPAR-α present in the initial segment of the gastrointestinal tract, such as stomach, duodenum, and jejunum. Importantly, these data were recently independently confirmed by another group (322). In conclusion, OEA is a new orally active anorectic agent that may possess potential as a new antiobesity drug.

V. Cannabinoid Receptor Antagonists as New Pharmacological Tools to Tackle Obesity and Obesity-Related Diseases

Section:

A. Emerging issues in the treatment of obesity and related diseases by cannabinoid antagonists

The whole body of data mentioned above highlights the role of the endocannabinoid system in feeding and energy balance regulation. Indeed, it was reasonable to hypothesize a therapeutic role for cannabinoid antagonists in the treatment of obesity. SR141716, also named rimonabant (commercialized as Acomplia), is now undergoing multicenter randomized, double-blind phase III trials to assess the effects on weight loss in obese patients with or without comorbidities with dyslipidemia and with type 2 diabetes (323). Moreover, the multitude of patents filed over the last few years claiming the synthesis of novel CB1 receptor antagonists reflects the intense competition in this area (123). Other compounds are under development, such as SLV-319 (Solvay, Weesp, The Netherlands) (129), which is undergoing phase I trials (323). However, at present, little is known about the results of these trials.

B. Clinical trial studies with rimonabant, the first CB1 receptor antagonist in clinical use to tackle obesity and obesity-related diseases

The CB1 receptor antagonist rimonabant was initially tested in humans not as an antiobesity drug but for its potential ability to reduce subjective intoxication and tachycardia in healthy subjects with a history of marijuana use or as an antipsychotic agent in schizophrenic patients. The first study showed that rimonabant was well tolerated by the participants even at a 90-mg dose (single oral dose). A significant dose-dependent blockade of marijuana effects was shown. However, the ability to reduce the intoxication induced by marijuana was very mild (130).

The results derived from the clinical trial in which rimonabant was tested to treat schizophrenia and schizoaffective disorders were not very satisfactory, because the effects of the drug in ameliorating clinical symptoms were not different from those obtained by placebo (324). However, in this trial, rimonabant treatment at 20 mg/d dosage was very well tolerated.

Bearing in mind the function of the endocannabinoid system in the mesolimbic rewarding system, rimonabant is also undergoing clinical trials as an aid to preventing the relapse of smoking cessation (323). Preliminary data from the STRATUS-US trial (smoking cessation in smokers motivated to quit) were recently presented at the 53rd Annual Scientific Session of the American College of Cardiology. The clinical study enrolled 787 smokers who received rimonabant at a dose of 5 or 20 mg or a placebo in a randomized fashion. The clinical trial lasted 10 wk, and the smokers were permitted to smoke during the first 2 wk but were asked to abstain from smoking after this period. The quit rate for subjects in the 20-mg rimonabant group was double that of the placebo group. In particular, the smokers characterized by overweight and obesity showed a relevant reduction in weight gain over the 10-wk treatment (325).

The most promising data seem to derive from rimonabant as a treatment for obesity. A phase II, 4-month, double blind, placebo-controlled study examined the effect of three different dosages of rimonabant (5, 10, or 20 mg/d) in obese patients with a body mass index between 30 and 40 kg/m2. Patients taking the 20-mg dose reported a weight loss of 4.4 kg in comparison to the 1.1-kg average in the placebo group. No significant adverse effects were noted. At the end of the treatment, weight loss was not maintained. However, the rebound in weight did not reach the pretreatment values (323). Another phase II, 7-d treatment, double-blind, placebo-controlled study was performed to evaluate hunger, calorie and fat intake. All these parameters were significantly reduced at the end of the short treatment, and the resulting average loss in body weight was 0.72 kg. The drug showed a good safety profile (323).

A large phase III trial named as RIO (rimonabant in obesity) was initiated in August 2001 including more than 6600 overweight or obese patients (323). All studies have already been concluded, and some of them are already reported in the literature (326, 327). Two of these studies, named RIO-North America and RIO-Europe, recruited obese and overweight patients with or without comorbidities who were treated for 2 yr with 5 or 20 mg rimonabant vs. placebo. The primary endpoints of the RIO-North America study were the absolute change in weight from baseline to 1 yr and the

prevention of weight regain after rerandomization (second year), whereas the main endpoint of the RIO-Europe study was the assessment of weight reduction by using the same dosages. Secondary endpoints of both studies were the number of weight responders and the changes in waist circumference, metabolic and lipid parameters, and the number of patients affected by the metabolic syndrome as defined by National Cholesterol Education Program's Adult Treatment Program III (NCEP-ATP III) criteria (328). RIO-Lipids and RIO-Diabetes are the other two clinical trials with rimonabant aimed at investigating the amelioration, after treatment with the CB1 receptor antagonist, of specific comorbidity factors associated with obesity or overweight such as hyperlipidemia and diabetes. In the RIO-Lipids study, presented by the American College of Cardiology in New Orleans in March 2004, 1036 obese patients characterized by lipid profile alterations and body mass index of 27–40 kg/m2 were randomized to double-blind treatment with either placebo or rimonabant 5 or 20 mg/d (326). All patients were required to follow a reduced calorie diet. After 1 yr of therapy, patients in the 20-mg dose group showed a loss of 8.8 kg compared with the 2-kg reduction in the patients treated with placebo. Rimonabant was associated with an important and significant reduction in waist circumference, tryglicerides, and C reactive protein, whereas a significant increase in HDL-cholesterol was found in the 20-mg treatment group compared with the group of patients undergoing placebo treatment. Forty-three percent of patients in the 20-mg treatment cohort lost more than 10% of their initial body weight compared with the 10.3% observed in the placebo group. The number of patients in the 20-mg rimonabant group classified as having metabolic syndrome (according to NCEP-ATP III criteria) decreased from 52.9 to 25.8% after 1 yr. Rimonabant was generally well tolerated, and the most frequently reported side effects were gastrointestinal and upper respiratory tract symptoms (326).

Similar data have been obtained by the ad interim analysis of the first year treatment in the RIO-Europe study (327, 329). More than 67% of patients who completed treatment with 20 mg rimonabant achieved 5% or more weight loss, whereas 39% achieved 10% or more weight loss. The pattern of weight loss appeared to be sustained for up to 36–40 wk. A concomitant reduction in waist circumference of about 9 cm was observed in patients treated with 20 mg rimonabant. A significant improvement of lipid and glycemic profile was also observed in this study in patients with 20 mg rimonabant, with a significant increase in HDL-cholesterol (22% vs. 14% in placebo-treated patients) and a concomitant reduction of triglycerides (6.8% vs. an increase of 8.3% in placebo-treated patients). As expected by studies in the animals described above, the study of Van Gaal et al. (327) demonstrated that rimonabant adds a further important and significant weight-independent effect on lipid parameters to the positive effects derived from weight loss and waist reduction. In fact, as determined by statistical analysis, the effect of 20 mg rimonabant on both HDL-cholesterol and tryglicerides at 12 months has been shown to be partly independent of weight loss, being 60% of the increase in HDL-cholesterol and 45% of the reduction in trygliceride accounted for by weight loss, and the remainder due to reasons not related to body weight changes (327). Although Van Gaal et al. (327) proposed that a rise in adiponectin might be responsible for these relevant positive changes in lipid profile, other mechanisms might enter into play. Full understanding of these still unknown modes of action is urgently needed to better characterize the ideal phenotype of obese patients to be targeted with CB1 receptor antagonist drugs.

Rimonabant treatment was well tolerated, and the most common adverse events experienced with 20 mg rimonabant were gastrointestinal symptoms such as nausea and diarrhea and mood disorders such as anxiety and depression. However, the effects were found to be mild, and the discontinuation rate due to these events was similar between patients taking 20 mg rimonabant or placebo. The genesis of these adverse events might be explained by bearing in mind that, as explained above, CB1 receptor plays a role in gastrointestinal motility and in HPA axis activation. Nausea and diarrhea on the one hand and anxiety and depression on the other hand might be due to CB1 receptor pharmacological blockade.

Concerning studies in humans, a very recent report (330) confirms, on a genetic basis, the possible association between the chronic pathological overactivation of the endocannabinoid system and the development of obesity. In fact, in a large cohort of Caucasian and black subjects, overweight and obesity have been found to be associated with a polymorphism in FAAH. This genetic variant predicts a substitution of threonine for a highly conserved proline residue (P129T). It has been observed that patients carrying this polymorphism may have approximately half the enzymatic activity of FAAH. This may lead to a reduced inactivation of AEA and, eventually, to an inappropriate chronic increase of endocannabinoid tone (330). In such a context, a recent work (60) showed increased circulating levels of AEA and 2-AG in obese women when compared with a lean control group. Moreover, in the same study, a marked down-regulation of FAAH gene expression in adipose tissue of obese women has been found, suggesting that the increased endocannabinoid levels may be secondary to decreased enzymatic degradation (60).

VI. Summary and Perspectives

Section:

A number of studies show that the endocannabinoid system profoundly influences both hormone secretion and metabolic processes. Animal models have represented the ideal tool for advancing the understanding of the mechanisms of these functions. However, the data derived from early studies were not always straightforward in the conclusions. The contradictory results had to be largely attributed to the heterogeneous variety of substances, dosages, and routes of administration used in each experimental model. Studies in humans with marijuana or Δ9-THC were even more contradictory in their conclusions, because no standardization of dose was used and no stringent criteria (i.e., randomization) of patient recruitment were defined in nearly all the experimental models.

However, the generation of CB1−/− mice and the introduction of CB1 receptor antagonists initially in animal models and later in humans provided a remarkable stimulus to better characterize the functions of the endocannabinoid system in the regulation of hormone secretion and metabolic processes (Fig. 3).

As a general conclusion, the endocannabinoid system appears to play a very important regulatory role in the secretion of hormones related to reproductive functions and to stress responses. These observations have led to some important clinical considerations. High levels of endocannabinoids seem to negatively affect reproduction by acting at different sites. It is therefore possible to speculate about a clinical use of CB1 receptor antagonists to ameliorate gonadotropin pulsatility or to improve fertilization capability. On the other hand, endocannabinoids are important modulators in the physiological response of the HPA axis during repetitive stress conditions and in pathological conditions, such as anxiety, phobias, depression, and posttraumatic stress disorders (16, 147). Moreover, the endocannabinoid system has been proposed as playing an important role in protection against neurotoxicity and, possibly, certain forms of epilepsy (115, 331, 332). Drugs presumed to increase endocannabinoid tone are therefore currently proposed as a new therapeutical frontier to treat anxiety-related disorders and neurodegenerative diseases (82). The use of drugs acting as antagonists of CB1 receptor should thus be carefully monitored when administered, for instance, to patients with anxiety traits, epilepsy, or neurodegenerative disorders.

The anecdotes regarding the orexigenic properties of marijuana have nowadays been substantiated by an impressive number of reports that make it possible to definitively include cannabinoids in the large family of orexigenic signals. This large body of data provided the basis to establish a novel approach to tackle obesity and related disorders by means, as strongly suggested by the clinical trials with rimonabant, of a CB1 receptor antagonist. During the last few years, it has become evident that multiple mechanisms of action, not solely limited to the CNS, are involved in the endocannabinoid-mediated control of food intake and energy balance. The full understanding of these modes of action may lead to the identification of the particular types of obesity where treatment with CB1 receptor antagonists work most efficiently. The potential clinical use of rimonabant will also help us to clarify how the endocannabinoid system affects the physiological functions and the pathological diseases related to hormonal secretion and energy balance.

Note Added in Proof

Section:

After this manuscript was accepted, the complete RIO-Lipids study was published (see Refs.326 and 333).

Section:

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Abbreviations: AEA, Arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl-glycerol; CB1−/−, mice lacking cannabinoid receptor type 1; CB1 receptor, cannabinoid receptor type 1; CB2 receptor, cannabinoid receptor type 2; CCK, cholecystokinin; CNS, central nervous system; D1, dopamine receptor type 1; D2, dopamine receptor type 2; DIO, diet-induced obesity; FAAH, fatty acid amide hydrolase; GABA, γ-aminobutyric acid; HDL, high-density lipoprotein; HPA, hypothalamus-pituitary-adrenal; IHC, immunohistochemistry; ISH, in situ hybridization; MCR4, melanocortin receptor type 4; OEA, oleoylethanolamide; PPAR, peroxisome proliferator-activated receptor; PRL, prolactin; PVN, paraventricular nucleus; RIO, rimonabant in obesity; Δ9-THC, Δ9-tetrahydrocannabinol; UCP, uncoupling protein; VTA, ventral tegmental area.

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Fig. 1. Expression of CB1 mRNA in soleus muscle of mice. RT-PCR was performed using 1 μg of total RNA extracted by phenol-chloroform method from soleus muscle as shown in Ref.58 . β-actin and CB1 mRNA expression. Lane 1, Wild-type 12-wk-old mice undergoing standard diet; lane 2, CB1−/− littermate mice undergoing standard diet; lane 3, C56BL/6 mice used as control undergoing standard diet; lane 4, C56BL/6 under high-fat diet for 2 months; lane +, positive control (hypothalamus); and lane W, negative control (PCR blank). Note the increased CB1 signal in muscle derived from mice on high-fat diet in comparison to the muscle derived from mice on standard diet.

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Fig. 2. Schematic representation of the main effects of CB1 on intracellular signaling cascades. Activation of CB1 leads to the stimulation of Gi/o proteins that, in turn, inhibits the adenylate cyclase-mediated conversion of ATP to cAMP. cAMP molecules can bind the regulatory subunits of protein kinase A (PKA) and cause the liberation of the catalytic subunits. Activated PKA can phosphorylate A-type potassium (K+A) channels, causing a decrease of the current. Given the negative effect of CB1 on adenylate cyclase, the final result is an activation of K+A channels. Gi/o activated by CB1 can also directly inhibit N- or P/Q-type Ca2+ channels and activate inwardly rectifying potassium (Kir) channels. These last two effects are controlled by protein kinase C (PKC), which, after activation, can phosphorylate CB1 in the third cytoplasmatic loop and uncouple the receptor from the ion channels. Activation of CB1 can also stimulate several intracellular kinases, such as focal adhesion kinase (FAK), phosphatidyl inositol-3-kinase (PI3-K) and its downstream effector protein kinase B (PKB)/AKT, ERKs, c-Jun N-terminal kinase (c-JNK), and p38 MAPK (p38). Stimulation of cytoplasmic kinases could also mediate the CB1-induced expression of the immediate early genes (IEG), such as the transcription factors c-fos, c-jun, and zif268, and the brain-derived neurotrophic factor (BDNF). Note that these events were described in different cellular systems and, therefore, they might not occur in the same cell types.

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Fig. 3. Actions of CB1 antagonists on the target organs involved in food intake and metabolic control. Schematic drawing illustrating the main sites of action of CB1 antagonists in the control of energy balance

TABLE 1. Overview of the cannabinoid action by CB1 activation in the various endocrine axes
TABLE 1. Overview of the cannabinoid action by CB1 activation in the various endocrine axes
Endocrine axis Cannabinoid actions Ref.
HPA axis Acute stimulation of CRH by CB1 agonists 135 136 137 138 139 140
Stimulation of the HPA by CB1 antagonists inducing a potentiation of stress-induced rise of the axis 143 144 146 147 148
Direct stimulation at the level of ACTH-producing cells (controversial data) 52 135 142
No studies on the direct effect at adrenal gland
Hypothalamus-pituitary-GH axis Inhibitory action through somatostatin activation 150 151 152 153 154
Hypothalamus-pituitary-thyroid axis Inhibition of T3 and T4 secretion by direct action at level of the thyroid 56
Hypothalamus-PRL axis Action at the level of PRL-producing cells 52 170 171
Inhibitory action through dopamine activation 153 161 162 168 169
Hypothalamus-pituitary-gonadal axis No effect on FSH 172
Inhibition of LH pulse through a multiple action on neuronal systems regulating GnRH secretion 54 165 172 173 174 175 176 183 184 185
Inhibition of testosterone and ovarian androgens 76 202 203 204
Inhibition of achrosome reaction 75 208 209
Inhibition of sperm fertilization and capacity 75 210

The ovulation blocking effect of cannabinoids: Structure-activity relationships

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Abstract

(1) Δ1-Tetrahydrocannabinol (Δ1-THC), the major psychoactive ingredient of cannabis, prevents ovulation when administered at 14:00 hr to pro-oestrous rats
(ed50= 0.98 mg/kg)
. Δ6-Tetrahydrocannabinol (Δ6-THC), a psychoactive isomer of Δ1-THC, was a less potent inhibitor of ovulation
(ed50= 3.76 mg/kg)
. However, the potent psychoactive 5′-(1″,2″ dimethylheptyl) (DMH) homologue of Δ6-THC (Δ6-THC-DMH) had a 60-fold higher ovulation-blocking potency
(ed50= 0.064 mg/kg)
than the parent compound and about 15 times higher potency than Δ1-THC. Moreover, the action of Δ6-THC-DMH was more prolonged than that of Δ1-THC, being expressed even when the compound was administered at 08:00 hr on the day of pro-oestrus, i.e. 6 hr before the critical period for neural triggering of ovulation. (2) (+) Cannabidiol [(+)CBD] was only weakly active as an ovulation blocker and 5,6-dihydro-7-hydroxy-2-isopropyl-5-methyl-9-pentyl-2,6-methano-2H-1-benzoxocin-4(3H)-one (C5 methyl equatorial) (compound 2a) and 5,6-dihydro-7-hydroxy-2-isopropyl-5-methyl-9-pentyl-2,6-methano-2H-1-benzoxocin-4(3H)-one (C5 methyl axial) (compound 3a) were inactive, but substitution of a DMH side chain for the amyl side chain of these three compounds resulted in significant enhancement of ovulation-inhibiting activity. (−)CBD and its DMH homologue did not affect ovulation in pro-oestrous rats. (3) (+) CBD-DMH (which showed ovulation inhibiting activity in the rat) has been found to be devoid of psychotropic activity when tested in rhesus monkeys (10 mg/kg). Apparently a separation between psychotropic and hormonal activity in the cannabinoids is possible.

Keywords
cannabinoids; ovulation-inhibition; psychotropic activity
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The cannabinoid system and immune modulation

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Abstract

Studies on the effects of marijuana smoking have evolved into the discovery and description of the endocannabinoid system. To date, this system is composed of two receptors, CB1 and CB2, and endogenous ligands including anandamide, 2-arachidonoyl glycerol, and others. CB1 receptors and ligands are found in the brain as well as immune and other peripheral tissues. Conversely, CB2 receptors and ligands are found primarily in the periphery, especially in immune cells. Cannabinoid receptors are G protein-coupled receptors, and they have been linked to signaling pathways and gene activities in common with this receptor family. In addition, cannabinoids have been shown to modulate a variety of immune cell functions in humans and animals and more recently, have been shown to modulate T helper cell development, chemotaxis, and tumor development. Many of these drug effects occur through cannabinoid receptor signaling mechanisms and the modulation of cytokines and other gene products. It appears the immunocannabinoid system is involved in regulating the brain-immune axis and might be exploited in future therapies for chronic diseases and immune deficiency.

• marijuana
• Th1 cells
• chemotaxis
• tumor
• G protein
• CB1

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INTRODUCTION

The normal immune system is modulated and regulated by foreign antigens that induce the production and secretion of a plethora of proteins from immune and nonimmune cells. These proteins include cytokines such as interleukin (IL)-2, chemokines such as monocyte chemotactic protein-1 [1], and surface receptors and other membrane proteins. In addition to these primary immune modulators, other humoral factors and metabolic products also modulate immunity. For example, prostaglandins [2], steroids [3], and histamine [4] are known to exert powerful effects on the quantitative and qualitative aspects of immunity. In addition, mediators of the brain-immune axis such as catecholamines [5], endogenous opioids [6], and cannabinoids [7 , 8] are also capable of modulating immune function. It is reasonable to speculate that these latter factors serve as secondary modulators, which when mobilized coincident with or shortly after first-line immune modulators such as lymphokines, increase or decrease immune activity. These secondary mediators are relatively simple in structure and therefore have been synthesized. This, along with the fact that the pharmacology of these agents in many cases has been thoroughly studied, makes these compounds good candidates for use in immunotherapy.

The medicinal uses of marijuana were described centuries ago for diseases such as asthma, migraine, pain, convulsions, and anxiety (reviewed in ref. [9]). More recently, emphasis has been placed on marijuana's putative, beneficial effects on appetite, glaucoma, spasticity in multiple sclerosis, pain, and inflammation [10]. Recent experimental evidence supports marijuana's therapeutic potential in some of these maladies [11]. The active plant ingredients in marijuana belong to the C21-cannabinoid compounds including the primary psychoactive compound, Δ9-tetrahydrocannabinol (THC). This cannabinoid along with others such as Δ8-THC, cannabidiol, and cannabinol, as well as chemical analogs, have been extensively studied over the years for their biological and therapeutic properties [8]. Some of the properties of these agents have included effects on immunity ranging from suppression of resistance to infection to enhancement of IL-1 production by macrophages. These early studies about the immunomodulating effects of these drugs have been the subject of previous overviews [7 , 12 13 14 15 16 17 18] and will not be reviewed here. Instead, we will briefly summarize the general features of the cannabinoid system and review recent findings on the structure and function of the cannabinoid system components in the immune system. For convenience, we will refer to this as the "immunocannabinoid" system.

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CANNABINOID SYSTEM

Marijuana cannabinoids, analogs, and endocannabinoids

The flowering tops and leaves of the marijuana plant contain many chemicals including the C21, tricyclic, cannabinoids, the most widely studied of these is THC, which is also the most potent in terms of psychoactivity (for review of structures, see ref. [8]). Cannabinoids are lipophilic and therefore difficult to work with; however, several were isolated in the 1940s, and by 1964, THC was isolated and synthesized [19]. Elucidating the structure allowed for the subsequent synthesis and testing of active analogs with varying cannabimimetic potencies leading to the pharmacological demonstration of cannabinoid receptors [9 , 20]. Of the many analogs synthesized, several have been used extensively over the years. One widely studied analog is the dimethylheptyl derivative of 11-hydroxy-Δ8-THC (HU210), which has a much higher affinity for cannabinoid receptors than THC (ref. [21];Table 1). In addition to the tricyclic cannabinoids, bicyclic analogs, such as CP55,940 [29], and aminoalkylindoles, such as R-(+)-WIN55,212 [30], have also been synthesized with cannabimimetic activity and with affinities higher than THC (Table 1) . All of these agents have been widely used in examining the biology of the cannabinoid system and as can be seen from Table 1 , these agents are relatively nonselective for cannabinoid receptors in that their Kis are similar for both. More recently, CB2-selective agonists have been synthesized, such as JWH-015, which displays a higher affinity for CB2 than for CB1 (ref. [31]; Table 1).

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Table 1.

Binding Affinities of Cannabinoid Receptor Ligands

Besides the classical agonists, such as THC, and nonclassical agonists, such as HU210, other ligand groups have been described. One of these, the so-called endocannabinoid group, is chemically eicosanoid in structure, and its members are derived from free arachidonic acid (ref. [32]; Fig. 1). The first of this group was isolated from pig brain and was determined to be an arachidonyl ethanolamide. This compound was named anandamide [33] and was shown in some systems to be relatively nonselective for either of the cannabinoid receptors (Table 1) . The existence of this endogenous ligand had been predicted by the discovery of cannabinoid receptors, and its demonstration established the existence of the endocannabinoid system of receptors and ligands. More recently, several other endocannabinoids have been isolated from tissues, such as 2-AG [26] and 2-AGE [34], the latter of which appears to be relatively selective for CB1. Anandamide and 2-AG are produced in brain as well as spleen and other peripheral tissues [35 , 36] and catabolized by the membrane-bound serine hydrolase, fatty acid amide hydrolase [37], and monoacylglycerol lipase [36], respectively. In addition to binding to cannabinoid receptors, anandamide also binds to vanilloid receptors, which are ligand-gated cation channels sensitive to capsaicin and related analogs [38].

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Figure 1.

The endocannabinoid system and cell function. An influx of Ca2+ activates phospholipases (PL), which drive the conversion of membrane arachidonic acid (AA) to endocannabinoids such as anandamide (ANA), 2-arachidonoylglycerol (2-AG), and 2-arachidonylglyceryl ether (2-AGE). The endocannabinoids can be taken up and metabolized by fatty acid amide hydrolase (FAAH) or can bind to CB1 or CB2. These are G protein-coupled receptors (GPCR) capable of signaling through G protein subunits, Gα, -β, and -γ, leading to a modulation of nitric oxide (NO) and adenylyl cyclase (AC). In neurons, this signaling can lead to a change in K+ and Ca2+ currents and secretion of transmitters such as γ-aminobutyric acid (GABA). In addition, CB1 and CB2 signaling can lead to the activation of many other factors, including PLC, protein kinase C (PKC), nuclear factor-κB (NF-κB), extracellular signal-regulated protein kinase (ERK), focal adhesion kinase (FAK), steroid receptor coactivator (Src), mitogen-activated protein kinase (MAPK), and MAPK kinase (MEK).

Another group of cannabinoid receptor ligands is the diarylpyrazole group, which has antagonist/inverse agonist activity when tested in a variety of tissues. The first of these described is the CB1-selective compound, SR141716A [23], which has potent antagonist activity for the CB1 receptor. The second described is the CB2-selective compound, SR144528, which has potent CB2 antagonism (ref. [28]; Table 1). Since the description of these two, other antagonists have been reported, and these have recently been reviewed [8].

Cannabinoid receptors

Pharmacological evidence initially supported the existence of cannabinoid receptors of high affinity in brain tissue and with specificity sufficient to distinguish different enantiomers of cannabinoid analogs [20 , 39]. Subsequent to this, the first receptor, CB1, was cloned from a rat brain cDNA library using a probe derived from the sequence of bovine substance-K receptor [40]. The translated sequence of the gene yielded a protein of 473 amino acids and the structure of a 7-transmembrane, GPCR. Since then, homologues of CB1 have been cloned from humans [41] and mice [42], displaying 97–99% amino acid sequence identity from one species to the other. CB1 mRNAs are expressed in brain, especially in the basal ganglia, as well as several tissues in the periphery including immune tissue (see below). Polymorphisms in the human gene have been reported, including a G-to-A silent mutation at position 1359 in the coding exon and an (AAT)n repeat polymorphism outside of the exon [43]. The significance of these changes in terms of gene function and disease is not clear at this time. The CB1 coding sequence is contained in a single exon, but other cDNA segments have been reported, such as a 5′-untranslated first-exon and a large 3′-untranslated sequence. The second receptor, CB2, was cloned from a cDNA library from the promyelocytic, human cell line, HL60 [44]. The protein encoded by this gene was reported to contain only 360 amino acids and showed only 44% identity with the human CB1 receptor. The mouse [45] and rat [46] CB2 genes also have been cloned and encode proteins of 347 and 410 amino acids, respectively. The variation in length among these proteins occurs in the carboxy end with the rat protein containing an extended carboxy terminus [46]. Amino acid identity of CB2 from species to species is lower than for CB1, and the mRNAs for CB2 are found in peripheral tissues, such as spleen, but not in brain and tissue, such as heart.

Function of cannabinoid system

Cannabinoid receptors are GPCR, and very early on, it was recognized that they were linked to inhibition of adenylyl cyclase (ref. [20]; Fig. 1). In addition, GPCR are known to be linked to a variety of other second messengers and signaling components [47 48 49], and ligation of CB1 and CB2 has been shown to not only suppress adenylyl cyclase but also to activate it [50], suggesting these receptors activate factors other than Gαi [51]. In addition to adenylyl cyclase, cannabinoid receptor ligation has been shown to activate PLC [52], which in turn, might activate diacylglycerol and inositol triphosphatase, increasing intracellular calcium and PKC. Ligation also has been shown to inhibit Ca2+ currents and activate K currents [53 , 54] and to increase NO [55] synthesis (Fig. 1) . Cannabinoid receptor ligation also has been shown to lead to the activation of transcription factors such as members of the MAPK [56] and NF-κB [57 , 58] families. From this and other evidence, it is clear that signaling through cannabinoid receptors and other GPCR involves numerous second messengers and signaling factors and that a variety of cellular and genetic activities are regulated by these kinds of receptors, including neuronal excitability [59] and IL-12 gene expression [49].

Cannabinoid receptors and ligands are found in the brain and peripheral tissues, and our current understanding of its function, at least in the brain, is beginning to take shape. The role of endocannabinoids and cannabinoid receptors in modulation of neurotransmitter release recently has been reviewed [59 60 61]. Several reports in 2001 showed that CB1 is located on presynaptic nerve terminals, that endocannabinoids are released from depolarized postsynaptic neurons, and that CB1 agonists and antagonists can modulate presynaptic neurotransmitter release [62 63 64]. Stimulation of the CB1 receptors was shown to suppress the release of neurotransmitters, such as GABA, thus allowing the cannabinoid system to turn down the activity of the GABA terminal in a retrograde manner back across the synapse. In the case of GABA release in hippocampal cells, the cannabinoid system appears to mediate depolarization-induced suppression of inhibition and may account for the inhibitory effects of cannabinoid ligands on memory and movement [60].

IMMUNE MODULATION

Cellular effects

Prior to the discovery of the cannabinoid receptors and the endocannabinoids, numerous studies examined the effects of marijuana smoking or THC on immune cell function. These studies have been extensively reviewed over the years [7 , 12 13 14 15 16 17 18] and support several conclusions. The first of these is that moderate marijuana smoking has little in the way of acute effects on systemic immunity but may have significant suppressive effects on the function of immune cells directly exposed to the smoke. For example, marijuana smoking was associated with suppressing the antimicrobial activity, cytokine production, and cytokine responsiveness of pulmonary alveolar macrophages [65 , 66]. However, regarding heavy or chronic use, sufficient studies have not been done to conclude that intense drug exposure is not without consequence on immune cell function. In fact, several studies suggest that long-term use in humans may be associated with increased incidence of infection [67 , 68], squamous cell carcinoma of the head and neck [69 , 70], and modulation of cannabinoid receptors in immune cells [71]; in nonhuman primates, heavy use may be associated with suppression of lymphocyte [72] and alveolar macrophage [73] function. Another conclusion from early studies is that treatment of mice and rats with THC or treatment of immune cell cultures from humans and rodents has, for the most part, a suppressive effect on T and B cells, natural killer (NK) cells, and macrophages. Various functions from cytotoxic T cell killing to antibody production by B cells to phagocytosis and killing by macrophages have been studied. Most functions were suppressed by drug treatment, and the effective drug concentrations in vitro were in the μM range. As this concentration range was higher than the reported receptor affinities, it was concluded by many that nonspecific effects of these lipophilic drugs on cell membranes primarily mediated the effects of cannabinoids on immune cell function. However, since the cloning of the receptors, it is now recognized that immune cells do express cannabinoid receptors and therefore, the modulating effects of cannabinoids are being reexamined in the context of cannabinoid receptor function. In addition, questions are being asked concerning the possible role of the immunocannabinoid system of receptors and ligands in the normal regulation of immunity.

Cannabinoid receptor expression in immune cells

By Northern blotting, the initial report on the cloning of CB1 failed to detect the expression of CB1 mRNA in tissues outside of the brain [40]. However, the cloning of the human gene demonstrated the message in testis tissue, but again, other tissues, including immune system tissues, were negative [41]. In 1992, Kaminski et al. [74] reported the demonstration of CB1 mRNA in mouse splenocytes using the more sensitive method of reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of receptors by these cells was also supported by pharmacological studies using cannabinoid agonist effects on in vitro antibody production by the splenocytes. Several agonists, such as THC, CP55,940, and HU210, suppressed antibody production in a dose-dependent manner when added to sheep red blood cell-stimulated splenocyte cultures. However, the affinity and efficacy of these agonists were lower with spleen cells than reported for behavioral studies and studies involving neural tissue, suggesting a lower density of surface receptors and variation from neural tissue in receptor coupling and affinity in immune cells. Also, as immune cells express CB2 receptors in addition to CB1 (see below), it is not clear whether CB1 or CB2 was being stimulated in these studies. Subsequent studies showed that CB1 mRNA levels varied among cell subpopulations. In human peripheral blood cells, message was reported to be most abundant in B cells, and the rank order was B cells > NK cells > polymorphonuclear neutrophils (PMNs) > CD8 cells > monocytes > CD4 cells [75]. The level of message also varied among different leukocyte cell lines; Jurkat cells were negative, and THP1 cells were highly positive [75]. CB1 message levels also varied in mouse immune subpopulations, and consistent with human cells, splenic B cells expressed more message than macrophages or T cells [76 77 78]. In addition to mouse splenocytes, CB1 message has been detected in rat brain microglia cells [79] and in human peripheral blood cell-derived dendritic cells (DC) [80]. We also have observed that CB1 and CB2 mRNAs are expressed in bone marrow-derived DC from mice (unpublished results).

Antibodies to CB1 have been produced and used to examine the expression of CB1 protein on immune cells. Rabbit polyclonal antiserum to a CB1–glutathione-S-transferase (GST) fusion protein demonstrated several immunoreactive proteins in membrane preparations from Jurkat and a CB1-positive neuroblastoma cell line. Proteins of 45, 60, 70, and 87 kDa were consistent with CB1 expression [81]. Similarly, a 58-kDa protein was demonstrated in membranes from rat microglia cells using an antiserum to CB1–GST fusion proteins [79], and proteins of 64 and 83 kDa were demonstrated in human DC [80]. We have prepared polyclonal rabbit antiserum to the 14 terminal amino acids of CB1 conjugated to keyhole limpet hemocyanin (KLH) and used this to analyze cell membrane proteins from mouse splenocyte immune cell subpopulations. Figure 2 shows the antiserum detected a major band at 80 kDa in splenocyte subpopulations with B cells and macrophages showing more protein than T/NK cells. In addition, the neuroblastoma cell line, N18TG2, showed several bands, and the predominant one was at 67 kDa, suggesting a variation in immunoreactive protein size between brain cells and immune cells. We also tested several commercial polyclonal antisera prepared to CB1 peptides. Figure 3A and 3B , shows these antibodies demonstrate major bands at 65 kDa (Company A) and 75 kDa (Company B) and that these bands are also the major ones demonstrated in the neuroblastoma cell line. Curiously, all three antisera (Fig. 3A 3B 3C) demonstrate analogous protein bands in spleen cell preparations from CB1 homozygous knockout mice provided by Dr. Andreas Zimmer and co-workers [82]. This could result from several factors. The first is that other proteins expressed in mouse immune and neural tissue share epitopes with the CB1 protein. A search of the mouse genome using the website <http://www.ensembl.org/> and the 14 amino acids of the amino terminal end of the mouse CB1 protein shows scores of identical matches of five amino acids or less in the translated portion of the genome, suggesting that these CB1 epitopes are shared by many proteins of the mouse. A second possibility stems from the fact that in the knockout mice, the CB1 gene is disrupted with the neor cassette in the middle of the single-coding exon, leaving short 5′ and 3′ portions of this exon intact [82]. In fact, mRNA containing the 5′ end of the coding exon up to position 93 (accession #U22948) can be detected by RT-PCR in splenocytes from these mice at a higher level than in wild-type mice (unpublished results). It is possible, therefore, that fusion proteins containing amino and carboxy fragments of CB1 plus the neor insert are expressed in these mice and are being detected by the antisera. We are currently testing antiserum to epitopes coded by the deleted portion of the CB1 gene to see whether it can detect proteins of comparable size in immune tissue from knockout and wild-type mice and N18 cells. It is possible that this antiserum will detect comparable bands in only wild-type and N18 cells, suggesting the possibility of expression of CB1 fusion proteins in knockout mice. At this time, the specificity of available anti-CB1 antibodies remains in question.

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Figure 2.
Western blotting of membrane proteins from splenocytes using anti-CB1 antibody. Affinity-purified, polyclonal antiserum to CB1 peptide (MKSILDGLADTTFR-C) was used (160 ng/ml) to probe membrane proteins from splenocyte subpopulations and N18TG2 neuroblastoma cells. Lane 1, N18 cells; lane 2, whole splenocytes; lane 3, nonadherent splenocytes; lane 4, adherent splenocytes; lane 5, T cells; lane 6, B cells.

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Figure 3.
Analysis of CB1 by Western blotting of splenocyte membrane proteins from wild-type and knockout mice. (A) Membrane proteins probed with anti-CB1 antibody from Company A: lane 1, N18TG2 neuroblastoma cells; lane 2, wild-type splenocytes; lane 3, wild-type splenocytes; lane 4, homozygous knockout splenocytes. (B) Membrane proteins probed with anti-CB1 antibody from Company B: lane 1, N18 cells; lane 2, wild-type splenocytes; lane 3, wild-type splenocytes; lane 4, homozygous knockout splenocytes. (C) Membrane proteins probed with antibody prepared as in Figure 2 : lane 1, N18 cells; lane 2, homozygous knockout splenocytes; lane 3, heterozygous knockout splenocytes; and lane 4, wild-type splenocytes.

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Unlike CB1, CB2 was cloned from a leukocyte cell line rather than brain tissue [44]. CB2 transcripts were detected in HL60 cells and rat spleen but not in other tissues such as thymus and brain. mRNA for the receptor was also detected by RT-PCR in macrophages from rat spleen but not in T cells [44]. CB2 mRNA expression was extended to human tissues, wherein by Northern blotting, it was demonstrated in only spleen and peripheral blood mononuclear cells (PBMC) and not in other tissues including brain; however, by RT-PCR, it was demonstrated in immune tissues including PBMC, thymus, spleen, and tonsils [83]. When examining PBMC subpopulations, CB2 message was in the rank order of B cells > NK cells > monocytes > PMNs > T cells. These and other studies suggested that CB2 mRNA was expressed primarily in immune cells but not in brain and that the message was most abundant in B cells [46 , 77 , 83 , 84]. Rodent peritoneal macrophages, cell lines, and microglia cells were also shown to be positive for CB2 mRNA when examined by mutational RT-PCR [85]. As in other studies, the CB2 message was more abundant than CB1 and was readily expressed in cortical primary microglia.
As with CB1, antibodies also have been used to examine the expression of CB2 protein on immune cells. Polyclonal antiserum to a C-terminal peptide of CB2 was used for immunofluorescence studies in human tonsillar tissue [83]. Immunoreactive tissue was highest in germinal centers, suggesting B cells or antigen-presenting cells (APCs) were expressing the receptor. Polyclonal antiserum produced to amino acids 320–336 of the mouse protein conjugated to KLH was used to examine CB2 protein expression in homogenates of cultured rat peritoneal macrophages and brain microglia [85]. The main immunoreactive protein in peritoneal macrophages was ~40 kDa, and in microglia preparations, the protein was 27 kDa. The reason for the relatively small size of the microglia protein was not clear but was suggested to be a result of a degradation product of the predicted 40-kDa protein or a variant form unique to microglia [85]. In addition to these studies, commercial antibodies were used to analyze CB2 protein in rat spleen and human blood-derived DC [80]. In these samples, immunoreactive bands of 39, 47, and 59 kDa were observed, although the reason for the multiple bands was not clear. As with anti-CB1 antibodies, the specificity and reliability still remain in question.
Modulation of cannabinoid receptor expression by cell activation
Immune cells frequently express new gene products when stimulated with antigens and other bioactive substances. This also appears to be the case with cannabinoid receptors. The initial suggestion of this came with the cloning of CB2, wherein it was shown that CB2 mRNA was increased in HL60 cells by treatment with agents such as phorbol myristate acetate (PMA) [44]. At basal levels, HL60 cells were negative for CB2 mRNA by Northern blotting but became positive by 24 h following 12-O-tetradecanoylphorbol 13-acetate treatment. Since this report, stimuli, ranging from lipopolysaccharide (LPS) to anti-CD40 antibodies (Fig. 4), have been shown to increase CB2 expression. For example, the level of CB2 mRNA and protein in human tonsillar B cells [86] and mRNA in mouse splenocytes [84] was increased following activation by anti-CD40 antibody, and CB2 expression was increased by IFN-γ in mouse macrophages and microglia [85]. In addition, we recently showed that chronic marijuana smoking increased the CB2 mRNA in PBMC [71], suggesting that drug exposure may cause a change in receptor expression in humans as has been reported with CB1 in animal models [87]. Stimulation of immune cells also leads to a suppression of CB2 expression (Fig. 4) . For example, the differentiation of B cells in tonsils was accompanied by decreased expression of CB2 mRNA and protein in centroblasts as opposed to virgin B cells [86], and the stimulation by LPS of mouse splenocytes, macrophages, rat microglia, and human DC resulted in decreased expression of CB2 message [80 , 84 , 85]. In addition, TGF-β treatment of cultured human peripheral blood lymphocytes led to a decrease in CB2 protein with increasing concentration of the cytokine [88].

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Figure 4.
CB1 and CB2 expression is increased or decreased following cell activation. Stimuli reported to increase CB1 are LPS, anti-CD40 antibody, PHA, and marijuana use, and agents suppressing CB1 are LPS, PMA/ionomycin (Io), and anti-CD3 antibody. Stimuli reported to increase CB2 are PMA, anti-CD40 antibody, interferon-γ (IFN-γ), and marijuana use, and agents suppressing CB2 are LPS, differentiation, and transforming growth factor-β (TGF-β). PHA, Phytohemagglutinin.
CB1 expression is also modulated in immune cells by various stimuli (Fig. 4) . Initially, it was reported that PHA treatment in Jurkat cells [81] and LPS treatment of the mouse macrophage cell line, RAW 264.7 [89], induced an increase in CB1 message within several hours following treatment. However, studies with other cell types such as rat microglia [85] and human DC [80] showed that LPS treatment decreased rather than increased mRNA. The results with mouse splenocytes were even more complex with PMA/ionomycin or anti-CD3 antibody treatment, causing a decrease in CB1 message, and anti-CD40 treatment caused an increase [78]. It also has been reported that chronic marijuana smoking led to increased CB1 mRNA in PBMC, suggesting that drug use not only increased CB2 (see above) but also CB1 receptor expression [71]. From these studies, it is likely that the level of CB1 and CB2 receptors is modulated like many other immune gene products to varying degrees in various immune cell subpopulations. Although a few general themes are emerging in this regard, additional studies are needed using purified cells and detection reagents to define receptor expression in the immunocannabinoid system.
Cannabinoid receptor signaling in immune cells
A number of studies have defined various signaling events associated with CB1 and CB2 (see Function of cannabinoid system section). Many of these results were obtained in receptor-transfected cell systems or primary cells other than immune cells. However, a few reports have examined signaling events in immune cells in response to cannabinoid treatment. For example, intracellular calcium mobilization was studied in concanavalin A (Con A)-treated mouse thymocytes using fluorescent indicators and drug treatment with THC [90]. Cytosolic-free calcium was increased by Con A treatment, and this effect was suppressed by treatment with THC. The suppression of cytosolic calcium mobilization was a result of a drug-induced inhibition of extracellular influx as well as inhibition of calcium release from intracellular stores [90]. THC was also shown to suppress forskolin-stimulated adenylyl cyclase activity in mouse spleen cells [91] and purified splenic T cells [77]. The suppression of adenylyl cyclase by THC and CP55,940 was shown to be attenuated by treatment with pertussis toxin, suggesting that the cannabinoids were acting through Gi proteins [92].
Members of the NF-κB family of proteins also have been shown to be modulated in immune cells. The NK-like cell line, NKB61A2 [93], when treated with THC, was shown to increase the transcription of the IL-2Rα gene and increase the nuclear level of NF-κB protein [58]. However, in the macrophage cell line, RAW 264.7, THC was shown to inhibit NF-κB activity in response to treatment with LPS [57], suggesting that the type of cannabinoid effect on this family of proteins may be cell type- and stimulus-dependent. Several other immune cell lines have been tested for signaling modulation following cannabinoid treatment. The HL60 cell line was treated with CP55,940 and was shown to express higher levels of MAPK and Krox-24 gene activation [56], suggesting these signaling properties were increased by the drugs. In addition, EL4.IL2 mouse lymphoma cells treated with cannabinol or THC showed a decrease in PKA activity, activated protein-1, and NF-activated T cells binding [94 , 95]; furthermore, treatment of splenocytes with cannabinol decreased ERK–MAPK activity [96]. From these results, it appears that cannabinoid treatment of immune cells leads to modulation of various cannabinoid receptor-signaling pathways. This fact, coupled with immune modulation effects of these agents, suggests that cannabinoids may modulate immune cell function through CB1 and CB2 signaling mechanisms.
Modulation of T helper (Th) cells
Th cells are powerful regulators of cell-mediated (Th1) and humoral (Th2) adaptive immunity. Regulation of the development of these cells and the link between innate and adaptive immunity are areas of intense investigation [97] to better understand how infectious agents and other environmental factors affect the processes fundamental to activating host immunity. It is now clear that in addition to microbial products, drugs and endogenous factors regulate the development of Th1 and Th2 cells (Fig. 5). For example, prostaglandins increase Th2 activity and decrease Th1 activity [2]. In addition, glucocorticoids [98], morphine [99], and certain chemokines [49] are reported to suppress Th1 immunity, and adrenergic agents such as norepinephrine increase Th1 immunity [100].

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Figure 5.
Drugs and endogenous factors regulate the development of Th cells. Naïve CD4 Th (Th0) receive immune signals and in addition, receive signals from drugs and endogenous factors in the selective differentiation to Th1 or Th2 cells. Th1 development is decreased (↓) by prostaglandins, glucocorticoids, morphine, chemokines, and cannabinoids and is increased (↑) by norepinephrine. In contrast, Th2 development is decreased (↓) by norepinephrine and increased (↑) by prostaglandins, glucocorticoids, morphine, chemokines, and cannabinoids.
Cannabinoids were observed to suppress cell-mediated immunity to Legionella pneumophila infection when injected into mice shortly before infection. In addition, immunoglobulin G1 antibodies to Legionella were also increased in drug-treated mice, suggesting that THC caused, besides a decrease in cell-mediated immunity, an increase in Th2 activity and therefore a change in the balance of Th1 and Th2 normally observed in this infection model [101]. Additional studies using this model showed that THC injection suppressed Th1 immunity by inhibiting the production of IFN-γ and IL-12 as well as the expression of IL-12 receptors, and in addition, drug treatment increased the expression of the Th2-promoting cytokine, IL-4 [102]. It was also demonstrated, using CB1 and CB2 antagonists, that both receptors were involved in this THC-induced Th cell biasing. Other laboratories have reported Th biasing effects of cannabinoids (Fig. 5) . Splenocytes taken from THC-injected mice and stimulated in vitro with Con A were shown to produce reduced amounts of IL-2 and IFN-γ [103] and Propionibacterium acnes-primed mice injected with LPS showed a decreased production of IL-12 and increase of IL-10 when pretreated with HU210 and other cannabinoids [104]. Biasing toward Th2 immunity was also reported in a murine tumor model [105]. Mice injected with tumor cells and THC showed enhanced tumor growth, depressed cell-mediated immunity, and increased Th2 activity such as production of IL-10 and TGF-β, and these effects were attenuated by treatment with the CB2 antagonist, SR144528. A similar shift to Th2 cytokines was demonstrated in response to THC in activated peripheral blood T cell cultures [106]. In drug-treated cultures, proliferation was inhibited along with Th1 cytokines; Th2 cytokines were increased, and the CB2 antagonist inhibited the effects [106]. There is now ample evidence that cannabinoids and other GPCR agonists can modulate the development of Th (Fig. 5) . In the case of cannabinoids, it is possible that CB1 and CB2 may be differentially expressed on different subpopulations of APCs and Th cells. This selective expression could lead to an increase in Th2 development and a decrease in the development of Th1 cells, resulting in decreased cell-mediated immunity and increased antibody immunity. Continued research on the distribution and function of cannabinoid receptors as well as the production of endocannabinoids by different immune cells will provide greater insight into these mechanisms.

Cannabinoids and chemotaxis

Chemokine receptors, as noted above, are GPCR. It appears that other receptors of this group such as those for opioids and cannabinoids also are linked to cell-migration gene programs. For example, opioids are chemotactic when added to cultures of human blood monocytes and neutrophils, and these drugs can cross-desensitize cells to chemotaxis in combination with chemokines such as CCL5 [107]. These and other results provided evidence that the process of heterologous desensitization was occurring among chemotactic receptors that have varying affinities for different ligands such as chemokines and opioids. Cannabinoids also appear to have chemotactic activity for various cell types. HL60 cells expressing CB2 receptors were shown to migrate in response to CP55,940, and the migration could be demonstrated in experiments measuring chemotaxis and chemokinesis [108]. Drug treatment was also shown to increase the production of various cytokines and chemokines. Other cell types of immune lineage have been shown to migrate in response to cannabinoids. Myeloid leukemia cells expressing CB2 receptors displayed chemotaxis and chemokinesis in response to 2-AG but not in response to a variety of other cannabimimetic agents, and the migration was enhanced in the presence of IL-3 or granulocyte macrophage-colony stimulating factor [109]. 2-AG was also chemotactic for mouse spleen cells, and it was concluded that a major function for the CB2 receptor on these cells was the regulation of migration [109]. Mouse microglia cells also were shown to migrate in response to 2-AG through a CB2-mediated mechanism, and this effect was antagonized by the nonpsychotropic cannabinoids, cannabinol and cannabidiol [110]. Finally, in contrast to these immune cell studies, nonimmune cell migration is suppressed in response to cannabinoids. Human umbilical vein endothelial cells, when precultured with cannabinoids for 18 h, showed reduced chemotaxis in response to lysophosphatidic acid [111]. The CB2 antagonist inhibited this effect, and cannabinoid treatment was shown to suppress other functions involved in angiogenesis. From these studies, it appears that cannabinoids are similar to opioids in modulating cell migration and suggest that at least one function of the immunocannabinoid system is to regulate migration of immune cells.

Cannabinoids and tumor suppression

Marijuana smoking has been related to an increased incidence of head and neck cancer (see Cellular effects section); however, paradoxically, cannabinoids have been shown to suppress tumor development. Anandamide and other cannabinoids induce apoptosis in human PBMC, and this was suggested to account for the drug-induced suppression of lymphocyte proliferation [112]. These drugs were also shown to induce apoptosis and decrease Bcl-2 in macrophages and splenocytes, and this was suggested to contribute to increased processing and release of IL-1 [113]. Anti-tumor effects also have been linked to cannabinoid induction of apoptosis. Established gliomas in rats regressed in response to intra-tumor injection of THC and WIN55,212 [114]. Treatment of C6 glioma cells in culture with cannabinoids induced apoptosis by a pathway involving cannabinoid receptors and discrete signaling pathways involving ceramide and ERK activation. Similar experiments were performed using the CB2-selective agonist, JWH-133 [115]. Again, regression of established C6 tumors was observed following intra-tumor injection. In addition, a CB2 antagonist inhibited the effects, and cultures of C6 cells were induced to apoptosis in response to treatment with cannabinoids [115]. In other studies, proliferation of C6 glioma cells was suppressed by cannabinoids, an effect mediated by cannabinoid and vanilloid receptors [116]; furthermore, a variety of human and mouse malignant immune cell lines were induced to apoptosis by cannabinoid treatment, were suppressed in vivo by cannabinoids, and were inhibited through CB2-mediated mechanisms [117]. From these studies, it appears that cannabinoids can induce apoptosis and the inhibition of tumors through CB1 and CB2 mechanisms, suggesting their potential as anti-cancer drugs.
In addition to effects mediated by apoptosis, cannabinoids appear to suppress tumor cell growth by several other mechanisms. One of these involves the suppression of prolactin receptors. Endocannabinoids and synthetic cannabinoids were observed to inhibit the proliferation of human breast cancer cells [118]. The effect was CB1-dependent and resulted from the down-regulation of the long form of the prolactin receptor. Subsequent studies showed that endocannabinoids such as anandamide suppress the receptor by inhibiting adenylyl cyclase and MAPK and also can inhibit nerve growth factor receptor by CB1- but not CB2-mediated mechanisms [119 , 120]. From this, it appears that in some tumor suppression systems, both cannabinoid receptors are not involved, as in the case of apoptosis, suggesting cannabinoids affect tumor growth by mechanisms other than programmed cell death. Finally, cannabinoids have been reported to suppress tumor growth in several other ways, such as inducing the accumulation of lipid droplets in the tumor cells [121] and the suppression of angiogenesis [111], again suggesting that these drugs have the potential to affect tumor growth in a variety of ways. In contrast, the CB2 receptor has been reported to have oncogenic activity by inhibiting neutrophil differentiation through MEK/ERK and phosphatidylinositol-3 kinase signaling pathways [109]. It appears that the effect of marijuana and cannabinoids on tumor growth and development is exceedingly complex, showing enhancing and suppressive effects. This would suggest that the application of these agents to tumor therapy should proceed slowly and with consideration of all possible outcomes.

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SUMMARY

There is increasing effort to define and understand the mechanisms of the brain-immune connection. Many immune cytokines and brain neurochemicals engage in cross-talk between the immune and brain systems, orchestrating the regulation of immunity and chronic inflammatory diseases of the brain. The immunocannabinoid system of receptors and endogenous ligands appears to be a component of the brain-immune connection, and the details of its structure and function are now being elucidated. Immune cells express CB2 receptors and most probably, CB1 receptors, but considerable work is still needed to understand the distribution of these receptors among the various immune subpopulations, and in addition, a greater understanding is needed to define the factors that regulate receptor expression. It also is apparent that cannabinoid receptor

signaling regulates the expression of various genes in immune cells; however, the scope and mechanisms of these gene activation programs await further definition. Cannabinoid receptors are GPCR, and the importance of this family of receptors in immune cell function is increasingly recognized. Regulatory functions are emerging for these receptors involving the mechanisms of Th cell biasing as well as chemotaxis of leukocytes and the regulation of apoptosis and tumor suppression. These developments involving the cannabinoid system and immune regulation suggest the possibility that the components of this system will be exploited in the future for therapy of chronic diseases and immune deficiencies. Furthermore, continued work in this area will lead to a better understanding of immune regulation in general and the brain-immune connection in particular.

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The biological mechanisms that control mood, anxiety, stress, fear, and other emotional behavior have been focused almost exclusively on the role of certain neurotransmitters called monoamines. While monoamines like dopamine, serotonin, and norepinephrine play an important role in controlling mood and anxiety, the vast majority of drug-based therapies that target those "monoaminergic" systems are limited in their efficacy.

More recently, research has begun to highlight other neurochemical systems, including cytokines, peptides, and bioactive lipids. By diving deeper into the science of bioactive lipids, we begin to unearth the potential role of the endocannabinoid system (ECS) in stress and fear responses.

The Endocannabinoid System's Role in the Human Body

The ECS has been described in our previous article as a complex physiologic network within the human body comprised of cannabinoid receptors (CB1 and CB2). There are endogenous cannabinoid compounds such as anandamide and 2-AG, and their respective enzymes responsible for maintaining balance in the system by regulating the synthesis and breakdown of the active endocannabinoid compounds.

Not surprisingly, cannabinoid receptors and the biochemical machinery necessary to synthesize and generate cannabinoids are present within areas of the brain known to control emotional behavior, mood, stress, and fear. These structures include the prefrontal cortex, amygdala, hippocampus, and periaqueductal gray (PAG) of the midbrain.

What Happens in the Brain When Cannabinoid Receptors Are Activated?

Preclinical studies in mice have clearly shown that activation of cannabinoid receptors within the PAG via direct injection of anandamide reduces panic and anxiety behavior, or the "fight or flight" phenomenon. Other studies using a similar model in animals have shown decreased fear-avoidant and conditioned responses to pain or punishment.

However, before we jump the gun and feel like we have fear, anxiety, and stress responses all figured out, it's not as simple as activating receptors through cannabinoid administration. Other animal studies utilizing systemic injections of Δ9-THC (not directly into the PAG of the midbrain) have demonstrated mixed responses, eliciting either anxiogenic (promoting increased levels of anxiety) or anxiolytic (decreasing levels of anxiety).

This bidirectional or paradoxical response appears to be dose-dependent, where lower doses seem to reduce stress, anxiety, and panic, while higher doses may promote increased feelings of stress, panic, and fear. These opposing effects are likely a result of the involvement of other neurotransmitter circuits within the brain that, when inhibited, actually create a hyper-excitable response of stress, anxiety, and fear.

Striking a Balance within the Cannabinoid System

To illustrate what neuroscientists currently feel is happening, think of being in a car with the brake pedal and accelerator. When the cannabinoid system is functioning to decrease fear, stress, and panic, the foot is being taken off of the accelerator in the areas of PAG-midbrain centers.

On the other hand, when dosing is too high, or there is an imbalance in the endocannabinoid system, whereby CB1 receptors are being over-activated in lieu of CB2, that's like taking the foot off the brake pedal, thereby removing inhibition that was normally in place and allowing the engine to rev higher—increased fear, anxiety, and stress from activation of other circuits in the brain that are normally applying the brake.

More recently, other preclinical research has demonstrated the important role of maintaining a healthy endocannabinoid system "tone" for improved stress resilience and reducing the residual post-traumatic anxiety, fear, and panic behaviors in mice that were chronically exposed to stress. Interestingly, the greater the exposure to stress, the greater the magnitude of the response to treatment (by improving the ECS and cannabinoid signaling).

Are Cannabinoid Compounds the "Gatekeepers" to Healthy Stress Levels?

One theory is that anadamide and 2-AG are acting as "gatekeepers" to keep the stress response and high emotional loads at bay and improving recovery to pre-stress levels once the stress burden is reduced to baseline. Moreover, there is evidence from preliminary human studies that disrupting endocannabinoid signaling and regulation has important impacts on the HPA (hypothalamic-pituitary-adrenal) axis by increasing signs of anxiety and depression.

There is an emerging role in the interplay between the immune system and the neuro-endocrine system, such that the field of psychoneuroimmunology has exploded with new basic and preclinical research over the past 5 years. The crosstalk between the immune system, cytokine signaling, and the nervous system influencing behavior, anxiety, and mood has been proposed by numerous preclinical studies. A number of recent studies have demonstrated a role of CB2 receptors classically associated with the immune system in anxiety and depression-related behavior in animal models and human clinical studies.

In this context, it's interesting to note that a balanced ECS appears to be pivotal to a healthy stress response and mitigating fear, anxiety, and panic that tend to accumulate with dysfunctional responses to stress.

CBD and Anxiety Reduction in Humans

A randomized, double-blind, placebo controlled trial in 15 humans demonstrated that up to 600 mg of CBD (cannabidiol) reduced measured anxiety compared to increased levels with a 10 mg dose of Δ9-THC. CBD appears to activate other receptors outside CB2, including 5HT1A and TRPV1, both of which are involved in the anxiolytic and mitigating panic/fear responses to stress.

The totality of the best available scientific evidence points to the importance of balancing the tone of the endocannabinoid system for supporting a healthy stress response. This preclinical and emerging human clinical data support the use of a CBD-rich or CBD-overweighted extract of cannabis for modulating fear, anxiety, and a healthy stress response.
Addressing Your CBD Questions
If you have questions about CBD, send them to social@leafly.com or share them via Facebook or Twitter. Our goal is to spread awareness of and education about hemp-derived CBD products, so send us your questions and we'll address them in future installments!

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From <http://www.calgarycmmc.com/huntingtonsdisease.htm>

An Introduction to Huntington's Disease
Huntington's Disease (HD) is a severely debilitating neurodegenerative disease caused by a genetic mutation which leads to creation of mutated "huntingtin" protein (normally, the huntingtin protein functions to preserve cells of the nervous system [neurons], especially in the brain). Although the exact mechanism is not fully known, the development of this mutated protein leads to destruction of neurons in the basal ganglia, a part of the brain involved in movement regulation, as well as other areas.
Patients with Huntington's Disease tend to experience dysfunction in movement, cognition, and normal psychiatric functioning, and symptoms include severe, involuntary writhing movements known as "chorea", dystonia (involuntary tightening of muscles which results in "repetitive or twisting movements"), progressing dementia characterized by deficits in maintaining cognitive flexibility, behavior awareness, and impulse control, mood swings, irritability, depression (many patients commit suicide as a result of this biologically- and psychologically-based symptom), and others. Symptoms ordinarily begin in the 30s-40s, and patients usually pass away within 10 to 20 years of symptom onset, often as a result of infection. There is no known cure for HD, although medications are available to manage symptoms.
Cannabinoid Therapy
There is a limited amount of information available on the potential for cannabinoid therapies as treatment for HD. However, a study using an animal model, published in Brain in 2011, found the following results:

1. Reduction of type 1 cannabinoid (CB1) receptors in the basal ganglia led to increases in Huntington's-like symptom in mice with the human mutant huntingtin ("HMH") exon 1.
2. Cannabinoid therapy reduced Huntington's-like symptoms in mice with the HMH exon 1 who also had intact CB1 receptors.
3. Inhibiting expression of the CB1 receptor in mice with the HMH exon 1 may lead to a predisposition for neuronal damage.
4. CB1 receptors may work to control expression of brain-derived neurotrophic factor (BDNF, which is important for neuronal preservation in HD) – this is partially evidenced by lower levels of BDNF in mice with a lack of CB1 receptors.

These results show that the cannabinoid receptors may play a significant role in the pathogenesis of HD. Further study will provide additional, important information on whether or not cannabinoid therapy may prove helpful in treatment of the disease.
Additionally, there is mounting evidence that cannabinoids have the potential to work as neuroprotective agents (i.e. those that protect destruction of neurons) through processes such as improvement of function of mitochondria and the activation, reduction of inflammation, and cellular debris clearance, and may therefore prove to be useful for treatment of Huntington's Disease via various mechanisms. Increased research will help us to explore and harness any potential neuroprotective effects of cannabinoids. Please click here for more information on the potential for cannabinoid medicine use in the treatment of neurodegenerative diseases.
Huntington's Disease Symptom Relief
While no evidence has been gathered evaluating the use of whole-plant cannabis as a treatment option for patients with HD, there is a significant amount of information on its effects on symptoms often experienced by patients with Huntington's Disease, such as pain (1, 2) caused by dystonia and sleep disturbance. Additionally, evidence for relief from symptoms experienced by patients with Huntington's Disease, such as depression and anxiety, has been discovered with the use of cannabinoid therapy. Cannabis also may provide relief to patients experiencing potential side effects of certain standard HD medications, such as restlessness, hyperexcitability (i.e. neurons are stimulated too frequently), and fatigue (i.e. tiredness, often of a long-term nature), depending on the strain/ratio/concentration of cannabis/cannabinoids used. For more information on the characteristics of different cannabis strains, find previous coverage of this topic on Medical Jane here.
Endocannabinoid System Findings
Shown below is a summary of information available on the endocannabinoid system in patients with Huntington's Disease from the review "The influence of cannabinoids on generic traits of neurodegeneration", Fagan & Campbell, 2014. Note: CB1 and CB2 receptors are those to which cannabinoids bind and thereby exert their effects, the "caudate nucleus, putamen, and globus pallidus" are parts of the basal ganglia, the "striatum" is comprised of the caudate nucleus and putamen, the "cortex" is the outermost layer of neuronal tissue of the brain, microglia are brain cells that clear away cellular debris, "AEA" is anandamide and "2AG" is 2-arachidonoylglycerol (both are endocannabinoids, which are cannabinoids found naturally in the body), "NAPE-PLD" is an enzyme that forms AEA from a phospholipid (fat) precursor called NAPE, "DGL" is diacylglycerol lipase (an enzyme involved in the formation of 2AG), "FAAH" is an enzyme that acts to increase levels of AEA, "MGL" is monoacylglycerol lipase, an enzyme that degrades triglycerides (fats)

From <http://www.medicaljane.com/2014/11/18/huntingtons-disease-and-medical-marijuana/>

Conclusion and Warning to Huntington's Disease Patients
Given the highly favorable safety profile of whole-plant cannabis, it may be a safe and useful additional therapy for patients with HD who are finding it difficult to manage certain symptoms treated or side effects caused by standard therapies. However, due to the current lack of research evaluating/anecdotal evidence corroborating the benefits of whole-plant cannabis on patients with Huntington's Disease, and to the potential for psychoactive strains to exacerbate psychological discomfort/disorders and psychosis during intoxication (which are symptoms already experienced by

patients with Huntington's Disease at certain stages of progression), the use of whole-plant cannabis with higher levels of THC may not be an optimal treatment option for many patients. Contemplation of use should be carefully considered with the guidance of a healthcare professional, and if the decision in support of cannabis treatment is made, treatment should be started slowly, constantly and closely monitored by a healthcare professional, and discontinued at the first sign of patient discomfort.
Increased research on cannabinoid medicine and modulation of the endocannabinoid system in relation to Huntington's Disease and other neurodegenerative diseases has the potential to not only lead to symptom management for patients, but also potentially to novel therapies which may help to prevent progression, and potentially initiation, of the disease.

For information on reasonable expectations and safety in considering whole-plant medical cannabis use, as well as how you can advocate to move cannabis out of the Schedule I controlled substance classification in order to increase research on phytocannabinoids in the United States, click here.

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Cannabinoids And Huntingtons Disease

March 25, 2013 by Truth Seeker 0 Comments

Nabilone Could Treat Chorea and Irritability in Huntington's Disease

Adrienne Curtis, B.A., B.Sc. and Hugh Rickards, M.D., M.R.C.Psych., Department of Psychiatry, University of Birmingham, Birmingham, United Kingdom

Letter:

SIR: Huntington's disease causes chorea and psychiatric abnormalities. Psychiatric symptoms were found in one study in 51 out of 52 patients.1 Dysphoria, agitation, irritability, apathy, and anxiety were found in above 50% of the patients sampled.

Many sources postulate that cannabinoids could have a beneficial effect on the symptoms of Huntington's disease, especially on choreatic movements.2–4 As well as providing possible symptomatic relief in Huntington's disease, there is also some evidence5 that cannabinoids might have a neuroprotective effect which could delay the onset of symptoms by delaying or preventing the death of striatal neurons. This neuroprotective effect has also been postulated by other sources.6–8

To date there are only two reports on the use of cannabinoids in Huntington's disease in the literature. Cannabidiol, a nonpsychotropic cannabinoid, had no effect on chorea severity in 15 patients.9 In one single patient, single dose, uncontrolled open clinical trial using nabilone, 1.5mg, the chorea increased significantly.10 We present a case of a female patient with irritability, which improved after the introduction of cannabis. This improvement was maintained by treatment with nabilone.

Case Report

The patient was a 43-year-old female who died in December 2003. She developed symptoms of Huntington's disease at the age of 24 and her husband gave up paid employment to care for her in 1990 when she was 30 years old. In 1995, he reported difficulties in caring for his wife. These difficulties were related to personality changes due to her illness. She increasingly resisted help from professionals, especially care assistants, and refused any suggestion of short-term respite care. She became disinhibited and frequently undressed herself and walked around naked inside and occasionally outside the house. She exhibited a number of dangerous behaviors, such as leaving taps running and fires burning, and leaving burning

cigarettes around. Her husband became concerned about the effect that the care for his chronically ill wife was having on his son, who was born in 1984.
The patient went into residential care in 1996. The patient's husband visited two or three times every week and he always took his wife out for a trip. These trips were difficult because of the patient's refusal to be strapped into the car or her wheelchair, which sometimes resulted in falls caused by violent choreic movements when he was unable to physically hold her in the chair because he was using his hands for some other purpose.
In 2001, he began to give his wife cannabis to smoke when he took her out on these regular trips. When he returned his wife to the nursing home after these visits the staff were aware of a significant difference in the patient. The cannabis appeared to improve her mood and she was calmer and more relaxed. Prior to the introduction of cannabis she was extremely impatient and would get angry if required to wait even a few minutes for a cigarette. After taking cannabis, she was able to wait a while without screaming and throwing things. The patient also willingly accepted the use of a car seat belt and wheelchair harness.
In December 2001, the local general practitioner prescribed a regimen of nabilone, a synthetic 9-keto cannabinoid, which the patient began taking, 1mg each day. The husband and the nursing home staff both reported improvements in behavior and reduction of chorea coinciding with the introduction of cannabis and maintained by daily taking nabilone.
Comment
This report has many limitations. It is a single case report and no measurements were taken at the time of the introduction of cannabis and nabilone. The information was obtained by interviewing the husband and staff from the care home in 2005. The symptoms of Huntington's disease do change over time and the movements are different in the later stages of the disease. However both the husband and the staff are sure that the introduction of cannabis was beneficial and greatly improved the patient's quality of life in her last years. There is need for further trials to establish the therapeutic use of cannabinoids in the symptomatic treatment of Huntington's disease.
ACKNOWLEDGMENTS
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From <https://www.420magazine.com/2013/03/cannabinoids-and-huntingtons-disease/>

Pharmacol Rep. 2010 Jan-Feb;62(1):1-14.
Huntington's disease: pathogenesis to animal models.
Kumar P1, Kalonia H, Kumar A.
Author information
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Abstract
Huntington's disease (HD) is an inherited genetic disorder, characterized by cognitive dysfunction and abnormal body movements called chorea. George Huntington, an Ohio physician, described the disease precisely in 1872. HD is a dominantly inherited disorder, characterized by progressive neurodegeneration of the striatum but also involves other regions, primarily the cerebral cortex. The mutation responsible for this fatal disease is an abnormally expanded and unstable CAG repeat within the coding region of the gene encoding the huntingtin protein. Various hypotheses have been put forward to explain the pathogenic mechanisms of mutant huntingtin-induced neuronal dysfunction and cell death. None of these hypotheses, however, offers a clear explanation; thus, it remains a topic of research interest. HD is considered to be an important disease, embodying many of the major themes in modern neuroscience, including molecular genetics, selective neuronal vulnerability, excitotoxicity, mitochondrial dysfunction, apoptosis and transcriptional dysregulation. A number of recent reports have concluded that oxidative stress plays a key role in HD pathogenesis. Although there is no specific treatment available to block disease progression, treatments are available to help in controlling the chorea symptoms. As animal models are the best tools to evaluate any therapeutic agent, there are also different animal models available, mimicking a few or a larger number of symptoms. Each model has its own advantages and limitations. The present review deals with the pathophysiology and various cascades contributing to HD pathogenesis and progression as well as drug targets, such as dopaminergic, gamma-amino butyric acid (GABA)ergic, glutamate adenosine receptor, peptidergic pathways, cannabinoid receptor, and adjuvant therapeutic drug targets such as oxidative stress and mitochondrial dysfunction that can be targeted for future experimental study. The present review also focuses on the animal models (behavioral and genetic) used to unravel pathogenetic mechanisms and the identification of novel drug targets.
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Cannabinoids and Huntington's Disease
March 4, 2015March 4, 2015 agmed2017 Literature SummariesHuntington's
An Introduction to Huntington's Disease
Huntington's Disease (HD) is a severely debilitating neurodegenerative disease caused by a genetic mutation which leads to creation of mutated "huntingtin" protein (normally, the huntingtin protein functions to preserve cells of the nervous system [neurons], especially in the brain). Although the exact mechanism is not fully known, the development of this mutated protein leads to destruction of neurons in the basal ganglia, a part of the brain involved in movement regulation, as well as other areas.
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Cannabinoid Therapy
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1. Reduction of type 1 cannabinoid (CB1) receptors in the basal ganglia led to increases in Huntington's-like symptom in mice with the human

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2. Cannabinoid therapy reduced Huntington’s-like symptoms in mice with the HMH exon 1 who also had intact CB1 receptors.
3. Inhibiting expression of the CB1 receptor in mice with the HMH exon 1 may lead to a predisposition for neuronal damage.
4. CB1 receptors may work to control expression of brain-derived neurotrophic factor (BDNF, which is important for neuronal preservation in HD) – this is partially evidenced by lower levels of BDNF in mice with a lack of CB1 receptors.
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Additionally, there is mounting evidence that cannabinoids have the potential to work as neuroprotective agents (i.e. those that protect destruction of neurons) through processes such as improvement of function of mitochondria and the activation, reduction of inflammation, and cellular debris clearance, and may therefore prove to be useful for treatment of Huntington’s Disease via various mechanisms. Increased research will help us to explore and harness any potential neuroprotective effects of cannabinoids. Please click here for more information on the potential for cannabinoid medicine use in the treatment of neurodegenerative diseases.
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Conclusion and Warning to Huntington’s Disease Patients
Given the highly favorable safety profile of whole-plant cannabis, it may be a safe and useful additional therapy for patients with HD who are finding it difficult to manage certain symptoms treated or side effects caused by standard therapies. However, due to the current lack of research evaluating/anecdotal evidence corroborating the benefits of whole-plant cannabis on patients with Huntington’s Disease, and to the potential for psychoactive strains to exacerbatepsychological discomfort/disorders and psychosis during intoxication (which are symptoms already experienced by patients with Huntington’s Disease at certain stages of progression), the use of whole-plant cannabis with higher levels of THC may not be an optimal treatment option for many patients. Contemplation of use should be carefully considered with the guidance of a healthcare professional, and if the decision in support of cannabis treatment is made, treatment should be started slowly, constantly and closely monitored by a healthcare professional, and discontinued at the first sign of patient discomfort.
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First received: December 29, 2011
Last updated: January 31, 2013
Last verified: January 2013
History of Changes

Purpose
Huntington's disease (HD) is a progressive neurodegenerative disorder, related to an abnormal expansion of CAG triplets in the huntingtin gene, characterized by motor, cognitive and behavioral abnormalities, without known effective symptomatic treatment and without known disease slowing strategy. The most severe neuropathological lesions observed in HD take place in the striatum, one brain area important in motor control and rich in cannabinoid receptors (CBR). CBR are subdivided in two classes: CB1R are located in neurons and play a role in neuronal function; CB2R in brain are located mostly in microglia and modulate neuroinflammation.
CBR disappear early in the course of HD, before there is a massive drop out of cells in the striatum. Cannabinoid transmission is also an early event in brains of animal models of HD. In R6/2 mice, which carry large CAG expansions and develop an early and severe HD phenotype the suppression of the CB1R gene further accelerate the development of a severe clinical syndrome and the characteristic brain inclusions and abnormalities of synaptic density. R6/2 treated mice treated with cannabinoids improve their clinical phenotype, their brain lesions, the synaptic density and the levels of BNDF, a neurotrophic factor which enhances survival and resistance of striatal neurons.
Preliminary studies of cannabinoids in patients with HD have shown that these compounds are safe in these patients. Those studies, however, did not show efficacy because 1) they were underpowered from the statistical point of view, 2) were performed with isolated pure cannabinoids, instead of the more physiological stimulation with a mixture of compounds, and 3) they did use insensitive clinical parameters instead of sensitive end points, such as pathogenically important biomarkers.
The investigators propose a phase II trial with combination of cannabinoids with evaluation of safety, by the profile of adverse events, and efficacy, according to changes of important biomarkers

Condition Intervention Phase
Huntington's Disease Drug: delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) Phase 2
Drug: Placebo

Study Type: Interventional
Study Design: Allocation: Randomized
Endpoint Classification: Safety Study
Intervention Model: Crossover Assignment
Masking: Double Blind (Subject, Investigator)
Primary Purpose: Treatment
Official Title: A Double Blind, Randomized, Cross Over, Placebo Controlled Phase 2 Clinical Trial to Asses Neuroprotection by Cannabinoids in Huntington's Disease
Resource links provided by NLM:
Genetics Home Reference related topics: chorea-acanthocytosis Huntington disease
MedlinePlus related topics: Huntington's Disease
Drug Information available for: Dronabinol
Genetic and Rare Diseases Information Center resources: Huntington Disease
U.S. FDA Resources

Further study details as provided by Fundacion para la Investigacion Biomedica del Hospital Universitario Ramon y Cajal:
Primary Outcome Measures:
• Serious Adverse Events reported [Time Frame: 8 months] [Designated as safety issue: No]
• Changes in the UHDRs Score [Time Frame: On week 4 and 12 of each period] [Designated as safety issue: No]
UHDRS scale scores from the following perspectives: motor, cognitive, psychiatric and functional.

Secondary Outcome Measures:
• Changes in the BDNF levels (Brain-derived Neurotrophic Factor), oxidative stress (due to mitochondrial dysfunction) and proinflammatory cytokines in plasma [Time Frame: Basal and on week 4 and 12 of each period] [Designated as safety issue: No]
• Changes in the BDNF levels (Brain-derived Neurotrophic Factor), oxidative stress (due to mitochondrial dysfunction) and proinflammatory cytokines in cerebrospinal fluid. [Time Frame: On week 12 of each period] [Designated as safety issue: No]

Enrollment: 25
Study Start Date: September 2011
Study Completion Date: June 2012
Primary Completion Date: June 2012 (Final data collection date for primary outcome measure)
Arms Assigned Interventions
Experimental: Sativex Drug: delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD)
Sativex 2.7 mg delta-9-tetrahydrocannabinol/2.5 mg cannabidiol Oromucosal Spray. One spray per day, up to a maximum of 12 sprays per day.

Placebo Comparator: Placebo Drug: Placebo
Placebo, One spray per day, up to a maximum of 12 sprays per day.

Eligibility

Ages Eligible for Study: 18 Years and older
Genders Eligible for Study: Both
Accepts Healthy Volunteers: No
Criteria
Inclusion Criteria:
1. Patients with HD
2. Older than 18 years.
3. Able to understand the study, to attend the study visits and to provide informed consent.
4. Stable baseline medication for at least 6 weeks prior to randomization.
5. Score in the UHDRS-motor from 5 to 50.
6. Good cognitive status (MMSE> 25) at the screening visit, with no evidence of major depression, at the discretion of the attending physician, and no evidence of psychosis.
7. Not consumers of products derived from marijuana.
Exclusion Criteria:
1. Pregnant or lactating women.
2. History of drug addition.
3. History of psychosis or with history of suicidal attempt.
4. Patients with diseases of the oral cavity that prevents the safe administration of the drug.
5. Patients in which drug administration is contraindicated according to the SmPC

Contacts and Locations
Choosing to participate in a study is an important personal decision. Talk with your doctor and family members or friends about deciding to join a study. To learn more about this study, you or your doctor may contact the study research staff using the Contacts provided below. For general information, see Learn About Clinical Studies.

Please refer to this study by its ClinicalTrials.gov identifier: NCT01502046

Locations
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More Information
No publications provided

Responsible Party: Fundacion para la Investigacion Biomedica del Hospital Universitario Ramon y Cajal
ClinicalTrials.gov Identifier: NCT01502046 History of Changes
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Study First Received: December 29, 2011
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Keywords provided by Fundacion para la Investigacion Biomedica del Hospital Universitario Ramon y Cajal:
Huntington's disease, cannabinoids

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Huntington Disease Dronabinol
Basal Ganglia Diseases Analgesics
Brain Diseases Analgesics, Non-Narcotic
Central Nervous System Diseases Cannabinoid Receptor Agonists
Chorea Cannabinoid Receptor Modulators
Cognition Disorders Central Nervous System Agents
Delirium, Dementia, Amnestic, Cognitive Disorders Hallucinogens
Dementia Hormones
Dyskinesias Hormones, Hormone Substitutes, and Hormone Antagonists
Genetic Diseases, Inborn Molecular Mechanisms of Pharmacological Action
Heredodegenerative Disorders, Nervous System Neurotransmitter Agents
Mental Disorders Peripheral Nervous System Agents
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Huntington's Disease

Huntington's Disease (HD) is an inherited degenerative brain disorder characterized by motor abnormalities and dementia produced by selective lesions in the cerebral cortex and, in particular, the striatum. There are presently no known conventional therapies available to alleviate HD symptoms or delay HD-associated striatal degeneration.

Although the administration of cannabidiol in HD patients provided little symptomatic relief compared to placebo in a single clinical trial,[1] more recent preclinical data indicates that cannabinoids may possess potential to moderate the advancement of the disease and similar neurodegenative disorders.[2-3]

Specifically, experimental data published in the Journal of Neuroscience Research in 2011 reported that the combined administration of the plant cannabinoids THC and CBD provide neuroprotection in rat models of Huntington's Disease. Authors reported, "[O]ur data demonstrate that a [one to one] combination of THC and CBD-enriched botanical extracts protected striatal neurons against ... toxicity." By contrast, the administration of individual, selective synthetic cannabinoid agonists did not produce similarly favorable outcomes.

Investigators concluded, "In our opinion, these data provide sufficient preclinical evidence to justify a clinical evaluation of [one to one THC to CBD] cannabis-based medicine ... as a neuroprotective agent capable of delaying disease progression in patients affected by HD, a disorder that is currently poorly managed in the clinic, prompting an urgent need for clinical trials with agents showing positive results in preclinical studies."[4]

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Sativex-like Combination of Phytocannabinoids is Neuroprotective in Malonate-Lesioned Rats, an Inflammatory Model of Huntington's Disease: Role of CB1 and CB2 Receptors

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Abstract

We have investigated whether a 1:1 combination of botanical extracts enriched in either Δ9-tetrahydrocannabinol (Δ9-THC) or cannabidiol (CBD), which are the main constituents of the cannabis-based medicine Sativex, is neuroprotective in Huntington's disease (HD), using an experimental model of this disease generated by unilateral lesions of the striatum with the mitochondrial complex II inhibitor malonate. This toxin damages striatal neurons by mechanisms that primarily involve apoptosis and microglial activation. We monitored the extent of this damage and the possible preservation of the striatal parenchyma by treatment with a Sativex-like combination of phytocannabinoids using different histological and biochemical markers. Results were as follows: (i) malonate increased the volume of edema measured by in vivo NMR imaging and the Sativex-like combination of phytocannabinoids partially reduced this increase; (ii) malonate reduced the number of Nissl-stained cells, while enhancing the number of degenerating cells stained with FluoroJade-B, and the Sativex-like combination of phytocannabinoids reversed both effects; (iii) malonate caused a strong glial activation (i.e., reactive microglia labeled with Iba-1, and astrogliosis labeled with GFAP) and the Sativex-like combination of phytocannabinoids attenuated both responses; and (iv) malonate increased the expression of inducible nitric oxide synthase and the neurotrophin IGF-1, and both responses were attenuated after the treatment with the Sativex-like combination of phytocannabinoids. We also wanted to establish whether targets within the endocannabinoid system (i.e., CB1 and CB2 receptors) are involved in the beneficial effects induced in this model by the Sativex-like combination of phytocannabinoids. This we did using selective antagonists for both receptor types (i.e., SR141716 and AM630) combined with the Sativex-like phytocannabinoid combination. Our results indicated that the effects of this combination are blocked by these antagonists and hence that they do result from an activation of both CB1 and CB2 receptors. In summary, this study provides preclinical evidence in

support of a beneficial effect of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying signs of disease progression in a proinflammatory model of HD, which adds to previous data obtained in models priming oxidative mechanisms of striatal injury. However, the interest here is that, in contrast with these previous data, we have now obtained evidence that both CB1 and CB2 receptors appear to be involved in the effects produced by a Sativex-like phytocannabinoid combination, thus stressing the broad-spectrum properties of Sativex that may combine activity at the CB1 and/or CB2 receptors with cannabinoid receptor-independent actions.

Keywords: Phytocannabinoids, cannabidiol, Δ9-tetrahydrocannabinol, CB1 and CB2 receptors, Huntington's disease, malonate, basal ganglia, neurodegeneration, neuroprotection

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by motor abnormalities, cognitive dysfunction, and psychiatric symptoms.1 The primary cause of the disease is a mutation in the huntingtin gene consisting of a CAG triplet repeat expansion translated into an abnormal polyglutamine tract in the amino-terminal portion of this protein that becomes toxic for striatal and cortical neuronal subpopulations.2 At present, there is no specific pharmacotherapy to alleviate motor and cognitive symptoms and/or to arrest/delay disease progression in HD. Thus, even though a few compounds have produced encouraging effects in preclinical studies (i.e., minocycline, coenzyme Q10, unsaturated fatty acids, inhibitors of histone deacetylases) none of the findings obtained in these studies have yet led on to the development of an effective medicine.3 Importantly, therefore, following on from an extensive preclinical evaluation using different experimental models of HD, clinical tests are now being performed with cannabinoids. The preclinical studies with cannabinoids demonstrated preservation of striatal neurons by several agonists against different cytotoxic stimuli that operate in HD pathogenesis,4,5 effects that were exerted through multiple mechanisms of action, some of which involve the activation of CB1 and/or CB2 receptors and others of which do not. For example, cannabinoids with antioxidant profile, that is, Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD), protected striatal neurons against toxicity caused by the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP) that primes oxidative injury.6,7 However, the activity of Δ9-THC at the CB1 and CB2 receptors enables this phytocannabinoid to induce neuroprotection in other experimental models, for example, the transgenic mouse model of HD, R6/2, in which the effects of Δ9-THC are likely produced through the activation of CB1 receptors8 and possibly through the activation of CB2 receptors too, as selective agonists of this receptor type preserved striatal neurons in this genetic model9 and also in malonate-lesioned rats,10 a model priming pro-inflammatory events. Selective agonists of CB18,11 and CB29 receptors also preserved striatal neurons in in vitro or in vivo excitotoxic models. By contrast, other studies showed no effects in R6/1 mice using Δ9-THC, the synthetic agonist HU-210 and the inhibitor of the endocannabinoid metabolism URB597.12 All these data suggest that the evaluation of cannabinoids as disease-modifying agents in patients should be necessarily conducted with a broad-spectrum cannabinoid or with combinations of various cannabinoids with different and complementary pharmacological profiles. Sativex, a cannabis-based medicine recently licensed for the treatment of spasticity and pain in multiple sclerosis patients,13,14 has an appropriate profile for HD as it can activate CB1 and CB2 receptors due to the presence of Δ9-THC, but it can also exert cannabinoid receptor-independent antioxidant properties due to Δ9-THC and, in particular, to CBD.15 We recently initiated some experiments with the combination of Δ9-THC and CBD botanical extracts present in Sativex in those animal models of HD, in which individual cannabinoid agonists have proved to be effective,6−11 with the objective of determining whether this mixture also works in these models. We have just published the first data obtained in rats subjected to 3NP intoxication,16 a model priming, as mentioned above, calpain activation and oxidative injury as major cytotoxic mechanisms, and in which pure Δ9-THC6 and CBD7 administered separately, have already been found to display neuroprotective properties. The Sativex-like combination of phytocannabinoids also preserved striatal neurons from death caused by 3NP intoxication, and this effect was, as expected, independent of CB1 and CB2 receptors.16 These findings prompted us to extend our research to rats lesioned with malonate, an acute model of HD in which striatal damage is produced primarily by apoptosis and glial activation/inflammatory events and in which selective CB2 receptor agonists have been shown to be effective.10 To this end, we lesioned rats with an intrastriatal injection of malonate and used these animals for two different sets of experiments. First, we examined the neuroprotective effects of a 1:1 combination of botanical extracts of Δ9-THC and CBD, a combination that approximates to the 1.0:0.93 mixture of these extracts that is present in Sativex. The level of neuroprotection was evaluated by measuring the following parameters: (i) the volume of edema measured by in vivo NMR imaging; (ii) the number of Nissl- and FluoroJade B-stained cells which correlate with the number of surviving and degenerating cells, respectively, in the striatal parenchyma; (iii) the presence of reactive microgliosis, by using Iba-1 immunohistochemistry, and astrogliosis labeled with GFAP immunostaining; and (iv) the expression of various biochemical markers that have been found previously to be altered in this and other HD models, i.e. inducible nitric oxide synthase (iNOS), the neurotrophin IGF-1 and the CB1 receptor.8,10,16,17 In the second set of experiments, we explored whether the neuroprotective effects observed in this model with the combination of Δ9-THC and CBD present in Sativex involved the activation of CB1 receptors, CB2 receptors, or both, using selective antagonists for these two receptors (SR141716 and AM630, respectively). For this second set of experiments, we monitored the number of Nissl-stained cells, since this constitutes a very selective and sensitive marker of malonate damage in the striatal parenchyma.

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Results and Discussion

Only a few clinical studies have been performed to determine whether cannabinoid compounds are efficacious in HD, and the results they have yielded are rather controversial.18−21 Possibly, the reason for such controversy is that these clinical studies concentrated more on HD symptoms rather than on disease progression. Recent animal studies, however, have demonstrated that combinations of different cannabinoids or the use of a broad-spectrum cannabinoid may delay disease progression by preserving striatal neurons from death in different animal models of HD, thus stressing again the need for new clinical studies directed now at testing whether the neuroprotective effects that certain cannabinoids induce in experimental models of HD8−11,16 are also produced in HD patients. As mentioned in the introduction, we recently initiated some experiments with a 1:1 combination of the Δ9-THC and CBD botanical extracts that are present in Sativex in those animal models of HD, in which individual cannabinoid agonists have been found to be protective,6−11 with the objective to determine whether this mixture also works in these models. We used first rats subjected to 3NP intoxication, a model priming calpain activation and oxidative injury as major cytotoxic mechanisms, and in which pure Δ9-THC6 and pure CBD7 administered by themselves, had displayed neuroprotective properties. The Sativex-like combination of phytocannabinoids also protected striatal neurons from death caused by 3NP intoxication and its effects were, as expected, independent of CB1 and CB2 receptors.16 In this Article,16 we already presented preliminary evidence that the Sativex-like combination of phytocannabinoids might also work in additional models of HD, as it was capable to attenuate malonate-induced iNOS up-regulation in rats lesioned with this neurotoxin, but this was the only parameter analyzed in these rats.16 The goal of the present study was to corroborate the potential of Sativex-like combination of phytocannabinoids in this model of HD, in which striatal damage is primarily produced by apoptosis and glial activation/inflammatory events,10 by measuring different histopathological and biochemical parameters that have been more directly related to striatal degeneration in this HD model.

Effects of Phytocannabinoids on Malonate-Induced Striatal Damage

The first objective was to establish that we could produce the characteristic striatal damage associated with the local administration of malonate.10 As expected, the administration of malonate damaged the striatal parenchyma as indicated by the increased edema volume recorded in in vivo NMR imaging ($F(2,19) = 8.86$, $p < 0.005$; see Figure Figure1),1), the reduction in the number of surviving cells determined by Nissl staining ($F(2,10) = 6.36$, $p < 0.05$; see Figure Figure2),2), the increase in the level of degenerating cells determined by FluoroJade B staining ($F(2,10) = 32.87$, $p < 0.0005$; see Figure Figure2),2), and the extent of reactive microgliosis labeled with Iba-1 immunohistochemistry ($F(2,12) = 4.652$, $p < 0.05$; see Figure Figure3)3) and astrogliosis labeled with GFAP immunohistochemistry ($F(2,11) = 9.25$, $p < 0.01$; see Figure Figure3).3). Malonate toxicity also affects the expression of different markers related to inflammation (i.e., iNOS: $F(2,11) = 5.78$, $p < 0.05$) and neurotrophins (i.e., IGF-1: $F(2,15) = 4.41$, $p < 0.05$) (see Figure Figure4).4). These changes, although characteristic of a specific experimental model of HD, have also been found in HD patients using CSF, blood cell, and post-mortem brain samples,22,23 thus supporting the idea that glial activation associated with inflammatory events is part in the pathogenic process that occurs in HD patients. Lastly, malonate toxicity is also associated with a reduction in the expression of CB1 receptors ($F(2,25) = 4.863$, $p < 0.05$; see Figure Figure4)4) an effect largely related to HD pathogenesis in different animal models.6,8,16

Figure 1

Volume of edema measured by in vivo NMR imaging procedures in the caudate-putamen of rats subjected to unilateral lesions with malonate and

receiving two injections of Δ9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio ...

Figure 2
Nissl and FluoroJade B staining measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid ...

Figure 3
Iba-1 and GFAP immunostainings measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid ...

Figure 4
Gene expression for iNOS, IGF-1, and CB1 receptors measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 ...

The administration of a 1:1 combination of Δ9-THC- and CBD-enriched botanical extracts that are present in Sativex attenuated, to a different extent, all these malonate-induced changes. For example, it slightly reduced the volume of edema measured with in vivo NMR imaging procedures 24 h after malonate application and 24 h before animal death (Figure (Figure1).1). These in vivo results correlated well with the data obtained after the histopathological analysis of animal brains. For example, the treatment with a Sativex-like combination of phytocannabinoids increased the number of Nissl-stained cells that had been reduced by malonate (see Figure Figure2),2), while reducing the high number of degenerating cells stained with FluoroJade-B (see Figure Figure2).2). In a similar way, the Sativex-like combination of phytocannabinoids attenuated the activation of glial elements caused by malonate (i.e., reactive microglia labeled with Iba-1, and astrogliosis labeled with GFAP; see Figure Figure3),3), which was particularly abundant and generalized along the whole striatal parenchyma in this experimental model. Lastly, the Sativex-like combination of phytocannabinoids attenuated the malonate-induced up-regulatory responses in iNOS (already described in ref (16)) and IGF-1 gene expression in the striatal parenchyma (see Figure Figure4),4), whereas it partially recovered the malonate-induced reduction in CB1 receptors (see Figure Figure4). These4). These findings support the notion that the combination of both phytocannabinoid-botanical extracts may be effective as a neuroprotective therapy in malonate-lesioned rats as it was previously found when individual cannabinoid agonists (i.e., HU-308) were used in the same HD model.10 In this study,10 however, we found that CBD administered alone was not active against malonate-induced striatal damage, but, according to the present data, its combination with Δ9-THC resulted in highly positive effects presumably by enhancing the activity of this last phytocannabinoid at the CB1 and CB2 receptors (see below). The rationale for such combination has been largely discussed in previous reviews.24

Involvement of CB1 and/or CB2 Receptors in the Beneficial Effects of Phytocannabinoids on Malonate-Induced Striatal Damage

In our previous study that used an experimental model of HD that primes oxidative injury and calpain activation as major causes of striatal damage, we detected protective effects of the Sativex-like combination of phytocannabinoids, that seemed to result from the antioxidant and cannabinoid receptor-independent properties of both phytocannabinoids rather than from any interaction with CB1 and/or CB2 receptors. We wanted to see if this was also the case in the experimental model of HD used in the present study. To this end, we conducted additional experiments with selective antagonists for CB1 and CB2 receptor types, that is, SR141716 and AM630, respectively, combined with the Sativex-like mixture of phytocannabinoids. The same or similar antagonists had been already tested after being administered alone in the same animal model of HD, showing no effect in the case of CB2 receptor blockade10 or even aggravating striatal damage in the case of SR141716.25 In our present study, the blockade of CB1 receptors with SR141716 or the blockade of CB2 receptors with AM630 reversed the phytocannabinoid-induced decrease in the ability of malonate to attenuate Nissl-staining of cells ($F(5,23) = 6.11$, $p < 0.005$; see Figure Figure5).5). Interestingly, this reversal was no greater when both antagonists were administered together (see Figure Figure5).5). However, it is important to note that, although SR141716 and AM630, both separately and together, reversed the effects of the Sativex-like combination of Δ9-THC- and CBD-enriched botanical extracts, the lowest number of Nissl-stained cells was observed after the treatment with AM630, administered alone or in combination with SR141716. This finding, together with our previously published data showing that CB2 receptors underwent an up-regulation in glial elements located in the striatal parenchyma after malonate,10 whereas CB1 receptors underwent a down-regulation in the same animal model in the present study (see Figure Figure4)4) and also in other models of HD,8,16 suggests that even though Δ9-THC can activate both CB1 and CB2 receptors, the CB2 receptor plays a greater role than the CB1 receptor in the protection from malonate-induced striatal damage that the Δ9-THC- and CBD-enriched botanical extracts produce.

Figure 5
Nissl staining measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent ...

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Conclusion

Collectively, this study provides preclinical evidence in support of a beneficial effect of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying disease progression in a proinflammatory model of HD, which adds to previous data obtained in models priming oxidative mechanisms of striatal injury. However, the interest here is that, in contrast to these previous data, we have now obtained evidence that both CB1 and CB2 receptors are likely to be involved in Sativex effects, thus stressing the broad-spectrum properties of this medicine that may combine activity at the CB1 and/or CB2 receptors with cannabinoid receptor-independent actions. Moreover, our previous data showing that, in this proinflammatory model of HD, CB2 receptors were up-regulated10 whereas CB1 receptors were down-regulated (also demonstrated here), as well as our observation that the protective effect of the Sativex-like combination of Δ9-THC- and CBD-enriched botanical extracts could be blocked by the selective CB2 receptor antagonist AM630, suggest that CB2 receptors play a particularly important role in this protective effect, a hypothesis that is also in concordance with the previous literature (see ref (26) for review). Lastly, given that HD is a disorder that is currently poorly managed in the clinic, the present data obtained with malonate-lesioned rats, together with previously published data obtained using an oxidative model of HD,16 provide a strong justification for mounting clinical trials with cannabinoids that produce positive effects in these preclinical models of HD.

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Materials and Methods

Animals and Surgery

Adult (12 week old; 350–400 g) male Sprague–Dawley rats (Harlan Ibérica, Barcelona, Spain) were used for the experiments. Animals were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1 °C) with free access to standard food and water. All experiments were conducted according to local and European rules (directive 86/609/EEC) and approved by the “Comité de Experimentación Animal” of our university. Rats were subjected to unilateral lesions of the striatum with the complex II inhibitor malonate, following a procedure described previously.10 Rats were injected stereotaxically (coordinates: +0.8 mm anterior, +2.9 mm lateral from the bregma, −4.5 mm ventral from the dura mater) into the left striatum with 2 M malonate (dissolved in PBS 0.1 M, pH = 7.4) in a volume of 1 μL. The contralateral striatum of each animal remained unaffected, when data generated in this experimental model are expressed as percent of the lesioned side over the corresponding nonlesioned side.

Treatments and Sampling

We followed the same procedure used in previous studies with cannabinoids in malonate-lesioned rats.10,25 Thus, 30 min before and 2 h after the intrastriatal injection of malonate, animals were treated with combinations of botanical extracts enriched with either Δ9-THC, kindly provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. (Δ9-THC botanical extract contains 67.1% Δ9-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids) or CBD, also provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. (CBD botanical extract contains 64.8% CBD, 2.3% Δ9-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids). The total dose of

cannabinoid administered was always 4.63 mg/kg (equivalent to 3 mg/kg of pure CBD + Δ9-THC), a dose within the range of effective doses of both compounds when they were administered in pure form in this and other experimental models of HD.8−11 This was also the effective dose in our previous study with Sativex-like combination of phytocannabinoids in another HD model.16 Cannabinoids were prepared in Tween 80-saline solution (1:16) and they were administered i.p. Malonate-lesioned rats administered with vehicle, as well as sham-operated animals, were also included in this experiment. In a further experiment, rats injected with malonate, following the same procedure described above, and also injected with the 1:1 combination of Δ9-THC- and CBD-enriched botanical extracts used in the previous experiment, were coadministered (10 min before each injection of Sativex-like combination) with the CB1 receptor antagonist SR141716 (1 mg/kg), kindly provided by Sanofi-Aventis (Montpellier, France) or the CB2 receptor blocker AM630 (1 mg/kg), purchased from Tocris (Biogen Científica, Madrid, Spain), both prepared in Tween 80-saline (1:16). In both experiments, animals were killed 48 h after the administration of malonate and their brains were rapidly removed, the two striata dissected and frozen separately in 2-methylbutane cooled in dry ice, and stored at −80 °C for subsequent qRT-PCR analysis. Animals to be used for histological analysis were transcardially perfused with saline followed by fresh 4% paraformaldehyde [in 0.1 M phosphate buffered-saline (PBS)], and their brains were collected and postfixed overnight at 4 °C, and then immersed in antifreeze solution and stored at −20 °C for Nissl and FluoroJade B staining and immunohistochemical analysis. In all experiments, at least 5–6 animals were used per experimental group.

In Vivo NMR Imaging Procedures

In the first experiment consisting of injections of Sativex-like combinations of phytocannabinoids to malonate-lesioned rats, animals from the three experimental groups were used, at 24 h after malonate lesion and 24 h before animal death, for in vivo NMR imaging studies in which the volume of striatal edema was calculated. Experiments were performed at the Nuclear Magnetic Resonance Centre of Complutense University (Madrid, Spain) using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany) operating at 4.7 T, equipped with a 12 cm actively shielded gradient system. Rats were anesthetized with oxygen:isoflurane and subsequently placed in prone position inside a cradle. The animal head was immobilized and placed underneath a 4 cm surface coil. A respiration sensor was used to control the animals. First global shimmer was assessed, and then three gradient-echo scout images in axial, sagittal, and coronal directions were acquired (TR/TE = 100/3.2 ms, matrix = 128 × 128). A 3D fast spin–echo experiment with axial slice orientation was subsequently performed using the following acquisition parameters: TR = 3000 ms, effective TE = 86.5 ms, NA = 2, FOV = 2.56 × 2.56 × 1.28 cm3, matrix size = 256 × 128 × 32. The reconstructed matrix size was 256 × 256 × 32. The total time of the acquisition experiment was 27 min.

Histological Analyses

Coronal brain sections (25 μm thick) were obtained with a vibratome and collected as floating slices at the level of the caudate-putamen. They were used for Nissl (see details in ref (27)) and FluoroJade B (see details in ref (10)) staining, which permitted determination of the effects of particular treatments on cell number, and for immunohistochemical analysis of Iba-1, a marker of microglial cells, and GFAP, a marker of astrocytes. For immunohistochemistry, sections were incubated overnight at 30 °C with (i) monoclonal antirat Iba-1 antibody (Wako, Neuss, Germany) used at 1:300, or (ii) monoclonal antirat GFAP (Sigma-Aldrich, Madrid, Spain) used at 1:400. After incubation with the corresponding primary antibody, sections were washed in 0.1 M PBS and incubated for 2 h at 37 °C with a mouse highly cross-adsorbed AlexaFluor 488 secondary antibody (Invitrogen, Carlsbad, CA) for GFAP immunostaining, and with a rabbit biotinylated secondary antibody (Sigma-Aldrich, Madrid, Spain) followed by incubation for 2 additional hours with streptavidin 488 (Molecular Probes, Paisley, U.K.) for Iba-1 immunostaining. Both secondary antibodies were used at 1:200. Negative control sections were obtained using the same protocol with omission of the primary antibody. All sections for each immunohistochemical procedure were processed at the same time and under the same conditions. A Nikon Eclipse 90i confocal microscope and a Nikon DXM 1200F camera were used for slide observation and photography, and all image processing, including cell counting, was done using ImageJ, the software developed and freely distributed by the U.S. National Institutes of Health (Bethesda, MD). For this purpose, multiple sections, selected from levels located approximately 200 μm from the middle of the lesion, were obtained from each brain and used to generate a mean value per subject.

Real Time qRT-PCR Analysis

Total RNA was isolated from striata using RNATidy reagent (AppliChem. Inc., Cheshire, CT). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm, whereas its integrity was confirmed in agarose geles. After genomic DNA was removed (to eliminate DNA contamination), single-stranded cDNA was synthesized from 1 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) to quantify mRNA levels for IGF-1 (ref Rn99999087_m1), iNOS (ref Rn00561646_m1), or CB1 receptor (ref Rn00562880_m1), using β-actin expression (ref Rn00667869_m1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), and the threshold cycle was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA).

Statistics

Data were assessed by one-way ANOVA, followed by the Student–Newman–Keuls test.

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Acknowledgments

Authors are indebted to Yolanda García-Movellán for administrative assistance.

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Glossary

Abbreviations

3-NP 3-nitropropionate
CBD cannabidiol
GFAP glial fibrillary acidic protein
HD Huntington's disease
IGF-1 insulin like growth factor-1
iNOS inducible nitric oxide synthase
PBS phosphate buffer saline
Δ9-THC Δ9-tetrahydrocannabinol

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Author Contributions

# Both authors have contributed equally to this work.

Go to:

Author Contributions

▽ Both authors shared the senior authorship of this study.

Go to:

Author Contributions

O.S., R.G.P., and J.F.-R. designed the experiments included in this Article, which were conducted by S.V. and V.S. and supervised by O.S. and J.F.-R. J.F.-R. wrote the different versions of the manuscript, which were revised by the other authors. All authors have approved the final version for submission.

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Notes

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Pharmaceuticals Ltd. These agencies had no further role in study design, the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.
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New Study: Cannabinoids Protect the Brain and Heart From Injury

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/ By Alan Gallindoss /

Cannabis is in the news again for its purported medicinal benefits, with researchers in Israel last week indicating it may help prevent trauma to the brain in certain circumstances and may also help with cardiac problems. A few months ago an English pharmaceutical company that manufactures cannabinoids announced it was developing a new treatment for epilepsy using them.

Prof. Yosef Sarne in the Adelson Center for the Biology of Addictive Diseases in the Department of Physiology and Pharmacology at Tel Aviv University says that cannabis has neuro-protective qualities. He has found that extremely low doses of tetrahydrocannabinol or THC- the psychoactive component of marijuana- can protect the brain from long-term cognitive damage in the wake of injury from hypoxia (lack of oxygen), seizures, or toxic drugs.

Previous studies focused on injecting high doses of THC within much shorter time frames- approximately just 30 minutes- before or after injury. Sarne's papers in Behavioural Brain Research and Experimental Brain Research say that even extremely low doses of THC- around 1,000 to 10,000 times less than that in a conventional marijuana cigarette – administered over a wide window of 1 to 7 days before or 1 to 3 days after injury can jump-start biochemical processes which protect brain cells and preserve cognitive function over time.

In the laboratory, researchers injected mice with a single low dose of THC either before or after exposing them to brain trauma. A control group of mice sustained brain injury but did not receive the THC treatment. When the mice were examined 3 to 7 weeks after initial injury, recipients of the THC treatment performed better in behavioral tests measuring learning and memory. Additionally, biochemical studies showed heightened amounts of neuroprotective chemicals in the treatment group compared to the control group.

Brain populate with cannabinoid neurotransmitters

This treatment, especially in view of the long time frame for administration and the low dosage, could be applicable to many cases of brain injury and be safer over time, Sarne says and he theorizes that ultra-low doses of THC induces minor damage to the brain, which may actually "precondition" the brain to protect it against more severe damage from injuries such as lack of oxygen, seizures or toxic drug exposure. In other words, it may act as a sort of vaccine against more traumatic harm.

Professor Raphael Mechoulam has authored hundreds of scientific papers on cannabinoid research as well as a book, Cannabinoids as Therapeutic Agents, which provided an early review of the research in this area. Dr. Mechoulam was President of the International Cannabinoid Research Society and has received numerous honors and awards for his outstanding contributions to the field. He is a member of the Israel Academy of Sciences, and among the numerous prizes that he has received for his work, is the highest national scientific prize in Israel- the Israel Prize.
In 1964, Dr. Mechoulam and colleague, Dr. Yehiel Gaoni, were the first to identify and synthesize delta-9-tetrahydrocannabinol (THC), the primary psychoactive compound in cannabis. Their discovery provided new insights into how the brain functions and opened the door to medical research exploring, not only the therapeutic potential of THC (marketed as Marinol in America), but other natural and synthetic cannabinoids as well.
Dr. Mechoulam, along with pharmacologist Dr. Habib Edery and colleagues, went on to isolate and elucidate the structures of most members of the cannabinoid group of compounds in the cannabis plant. Twenty-eight years after discovering THC, in 1992, Dr. Mechoulam, along with Dr. William Devane and Dr. Lumir Hanus, identified the brain's first endogenous cannabinoid (or endocannabinoid)—the brain's natural version of THC—which they named "anandamide," from the Sanskrit word "ananda," which means "eternal bliss" or "supreme joy."
Research since revealed that the brain is richly populated with cannabinoid neurotransmitters and receptors. Just as the active compound in opium, morphine, led to the discovery of the endogenous morphine or endorphin system in the brain, research investigating the active compound in cannabis, THC, led to the discovery of the brain's endocannabinoid system. Later Dr. Mechoulam and colleagues identified the THC metabolites and, more recently, along with Dr. Lumir Hanus and Dr. Shimon Ben-Shabat, he discovered a second endocannabinoid known as 2-arachidonylglycerol (2-AG). These findings have profoundly advanced our understanding of cannabinoid systems.

Cannabinoids in Epilepsy Treatment
The English cannabinoid drug maker GW Pharmaceuticals announced in September 2012 it was planning to use a new component of the plant to create a treatment for epilepsy to add to its growing use as an existing treatment for multiple sclerosis and pain relief for cancer patients. This follows the publications of research by the University of Reading into a previously largely ignored substance "cannabidvarin" which occurs naturally in cannabis.
Scientists found that cannabidvarin works against epileptics' convulsions, but unlike other cannabis-based treatments does not make users "high".
Dr Stephen Wright, the research and development director at GW, said: "These results further underscore the potential of naturally derived cannabinoids as medicines to treat a range of diseases.

Tikun Olam
There is a government-approved farm tucked away in the Galilee hills in northern Israel that grows medical marijuana; the farm is called Tikn Olam. One variety they can grow there is said to have the strongest psychoactive effect of any cannabis in the world; while another variety, rich in anti-inflammatory properties, will not get you high at all but may be much more useful for medicinal purposes.
Casual consumption of marijuana is illegal in Israel, as in other countries, but farms like this one exist at the edge of the discussion on the legality, benefits and risks of medicinal cannabis. Its staff members wear white lab coats, its grow-ops are carefully managed and are protected 24/7 by security cameras and guards.
In Israel special licenses issued by the Ministry of Health now permit thousands of patients to receive medical marijuana, and some government officials have even praised the country's state of the art in the field as an example of its pioneering and innovation.
In the 1960s, Professor Raphael Mechoulam and his colleague Yechiel Gaoni at the Weizmann Institute of Science were the first to identify THC. Later, Professor Mechoulam deciphered the cannabinoids native to the brain. Tikkun Olam, a professor emerita of immunology at the Hebrew University of Jerusalem, has studied another main constituent of cannabis, cannabidiol, or CBD, considered a powerful anti-inflammatory and anti-anxiety agent.
In 2009, only about 400 Israelis were licensed to receive the substance; today, that number has risen to over 10,000. Working with Hebrew University researchers, the farm has also developed a version in capsule form, which would make exporting the drug more practical, should the law allow it.
In January Professor Mechoulam, now 82, said in an interview with the New York Times that he had been urging producers over the years to grow cannabis with less THC and more CBD, something in which non-medical marijuana growers had little interest. He said what Tikkun Olam had done did not advance theoretical science but was "a very practical development."
In Israel, he said, research in the field is "definitely a work in progress," and he cautioned, "Science is not a 100-meters Olympic race; it is not who is first on the line that is important." The real advances, he said, are being made not on farms but in laboratories around the world, including in the United States and Europe. The professor, who collaborates with many teams abroad, said that chances were that in the next few years well defined mixtures of the compounds, refined into something more like a medical drug, would replace today's medicinal marijuana.
In the meantime, he said, offering products with different levels of key ingredients, as Tikkun Olam does, "is going towards personalized medicine."
Yet experts say that many medical professionals in Israel remain skeptical and are reluctant to encourage patients to use marijuana, be it because of conservatism or a lack of knowledge about its potential benefits. Obtaining a personal-use license from the Health Ministry for a patient requires a special effort by doctors and can take more time than some patients have.
For centuries marijuana had been used for medical purposes and it first became a legally prescribed drug nation-wide in the United State only in the nineteen thirties in the aftermath of the final repeal of the prohibition of alcohol consumption in 1933. The Assistant Prohibition Commissioner who had been in charge of enforcing prohibition, Harry J. Anslinger, was widely admired as an incorruptible figure, and in 1930 he became the First Commissioner of the US Treasury Department's Federal Bureau of Narcotics. He then became both chief lobbyist to Congress and the White House, and subsequently chief enforcer, for marijuana regulation in the United Sates as a Class A illegal substance.
Of course dangers of smoking marijuana remain, as they do for smoking generally, and it remains a developing field with arguments on both sides of the debate.
Read more about: Brain, cannabinoid, Cannabis, Dr Stephen Wright, Dr. Habib Edery, Dr. Lumir Hanus, Dr. William Devane, Harry J. Anslinger, HEART, International Cannabinoid Research Society, Israel, Marijuana, New Study, Prof. Yosef Sarne, research, Tel Aviv University, Tetrahydrocannabinol, THC, Tikkun Olam, treatment

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• Ulcerative Colitis -- Undiagnosed
• Celiac Disease -- Undiagnosed
• Heartburn -- Undiagnosed
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• Ulcerative Colitis -- Rare Types
• Celiac Disease -- Rare Types
• Inflammatory Bowel Disease -- Rare Types
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The US based website ClinicalTrials.gov lists information on both federally and privately supported clinical trials using human volunteers.
Some of the clinical trials listed on ClinicalTrials.gov for Gastrointestinal disorders include:
• Phase II Pilot Study to Compare the Bioavailability of Buffered, Enteric-Coated Ursodiol With Unmodified Ursodiol for Chronic Cholestatic Liver Disease and Cystic Fibrosis-Associated Liver Disease - This study is no longer recruiting patients (Current: 23 Nov 2006) - ursodiol
• The Safety and Effectiveness of Ganciclovir in the Prevention of Cytomegalovirus (CMV) of the Eyes and Disease of the Stomach and Intestines in Patients With HIV - This study is no longer recruiting patients (Current: 23 Nov 2006) - Ganciclovir
• A Randomized, Double-Blind, Placebo Controlled Trial Evaluating the Impact of Nystatin on the Development of Oral Irritation in Patients Receiving High Dose Intravenous Interleukin-2 - This study has been completed (Current: 23 Nov 2006)
• Phase III Multicenter, Open-Label, Randomized Trial of Induction Versus Induction Plus Maintenance Foscarnet (Foscavir) Therapy for Gastrointestinal CMV Disease - This study has been completed (Current: 23 Nov 2006) - Foscarnet sodium
• Regulation of Human Intestinal Bile Acid Transport in Health and Disease - This study has been completed (Current: 23 Nov 2006)
• more trials...»
See full list of 1718 Clinical Trials for Gastrointestinal disorders
User Interactive Forums
Read about other experiences, ask a question about Gastrointestinal disorders, or answer someone else's question, on our message boards:
• I can not get a diagnosis. Please help.
• Tell us your medical story.
• Share your misdiagnosis story.
• What is the best treatment for this?
• See all the forums.
Definitions of Gastrointestinal disorders:
Illness caused by poisonous or contaminated food - (Source - WordNet 2.1)
Contents for Gastrointestinal disorders:
• Gastrointestinal disorders
• What is Gastrointestinal disorders?
• Videos related to Gastrointestinal disorders

• Types of Gastrointestinal disorders
• Causes of Gastrointestinal disorders
• Risk Factors for Gastrointestinal disorders
• Symptoms of Gastrointestinal disorders
• Diagnostic Tests for Gastrointestinal disorders
• Home Testing and Gastrointestinal disorders
• Signs of Gastrointestinal disorders
• Complications of Gastrointestinal disorders
• Misdiagnosis of Gastrointestinal disorders
• Misdiagnosis of Underlying Causes of Gastrointestinal disorders
• Medical Malpractice and Gastrointestinal disorders
• Treatments for Gastrointestinal disorders
• Doctors and Medical Specialists for Gastrointestinal disorders
• Cure Research for Gastrointestinal disorders
• Medical News Summaries About Gastrointestinal disorders
• Hospital Statistics for Gastrointestinal disorders
• Articles about Gastrointestinal disorders
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• Disease & Treatments Search
• Misdiagnosis Center
• Full list of interesting articles
Forums & Message Boards
• Ask or answer a question at the Boards:
○ I cannot get a diagnosis. Please help.
○ Tell us your medical story.
○ Share your misdiagnosis story.
○ What is the best treatment for my condition?
○ See all the Boards.

From <http://www.rightdiagnosis.com/g/gastrointestinal_disorders/intro.htm>

Marijuana and Treatment of Digestive Disorders

Licensed users and growers of medical marijuana know that the plant can be used to treat a variety of diseases and symptoms related to the gastrointestinal system. This isn't a bunch of stupid stoners trying to find a medical excuse to get high, folks, this is the real deal- the herb heals! The use of medical marijuana to treat digestive system problems is time-tested, and is usually a safer intervention than the use of pharmaceutical drugs or surgery.

Chron's Disease, Irritable Bowel Syndrome (IBS), ulcers, constipation, diarrhea, nausea, anorexia, bulimia, acid reflux, and appetite loss are some of the diseases and conditions that medical marijuana patients have treated using medical marijuana.

Studies indicate that cannabinoids in marijuana bind with cannabinoid receptors in the digestive tract, especially the small and large intestine, causing muscle relaxation, reduction of inflammation, analgesia, increased nerve-muscle coordination, anti-emesis, and relief of spasms such as those that cause nausea.

Cannabis is also an adaptogenic immune system modulator that can increase or decrease immune systems function in ways that almost always contribute to healthier outcomes.

Cannabis is unique among medicines because it has a comprehensive range of actions that can alleviate several symptoms by altering how the body and brain communicate, and how the self perceives its internal organs and systems.

The self-feedback loop that produces nausea in some circumstances, which includes a feeling of dizziness that circles back on itself to produce fear, tension and more dizziness, is often defeated by cannabis.

Indeed, research shows that cannabis can relieve symptoms in unique ways that no other medicine can duplicate.

Medical benefits of marijuana for people with gastrointestinal disorders were backed up by the United States Institute of Medicine medical marijuana study. According to the Institute, "For patients who suffer simultaneously from severe pain, nausea, and appetite loss, cannabinoid drugs might offer broad-spectrum relief not found in any other single medication."

Medical cannabis has been a blessing for people with Irritable Bowel Syndrome and Inflammatory Bowel Disease. These diseases cause diarrhea or constipation, nausea, and intestinal inflammation, pain and the inability of the digestive system to absorb nutrients.

Irritable Bowel Syndrome (IBS) can cause pain, bloating, flatulence, cramps, spasms, motility loss, constipation, and diarrhea.

Inflammatory Bowel Disease (IBD) is a term that describes Ulcerative Colitis and Crohn's Disease. Ulcerative colitis inflames the lining of the large intestine. Crohn's disease causes inflammation of the lining and wall of the large and/or small intestine. The typical symptoms of Crohn's are diarrhea, weight loss, abdominal pain, rectal bleeding and fever. Crohn's can cause intestinal blockages and ulcerations that might require surgery.

Ulcerative Colitis can cause abdominal cramps, sharp pain, low energy, weight loss, arthritic symptoms, eye problems, and liver disease.

These disorders can be crippling. In extreme cases, they may result in long-term hospitalization or surgery. The unique ability of medical cannabis to alleviate most of these symptoms is becoming more widely known in the medical community.

Recreational and medical marijuana users have long known that cannabis has an effect of appetite. The increase in appetite that often accompanies cannabis use has been called "the munchies."

Scientists studying this phenomenon note that it probably involves blood sugar levels and other physiological markers affected by cannabis.

Many professional studies have shown that cannabis stimulates appetite and weight gain. Researchers say that medical cannabis users have to be careful to moderate their intake of carbohydrates and sugars when they are responding to the munchies. It is best to eat less carbos and sugars and eat more organic vegetables, fruits and protein, rather than to pig out on candy or ice cream.

Cannabis helps combat cramping that accompanies many GI disorders because cannabinoids relax contractions of the smooth muscle of the intestines. Research shows that the body's own cannabinoids, known as anandamides, affect neurological systems that control the gastrointestinal system. External and internal cannabinoids strongly control gastrointestinal motility and inflammation. They also have the ability to decrease

gastrointestinal fluid secretion and inflammation. This means that cannabis can be useful to stop ulcers and other syndromes.
The chronic pain and spasms that accompany many gastrointestinal disorders are a life hindrance to those who suffer from IBS and other diseases. Medical cannabis is a very effective pain reliever. It blocks spinal, peripheral and gastrointestinal mechanisms that promote pain in IBS and related disorders. It also can be used against gastroesophageal reflux disease (acid reflux). When acid reflux occurs, gastric acids attack the esophagus. The pharmaceutical medicines that doctors prescribe for this condition are in some ways as bad as the condition itself. They prescribe drugs like atropine, for example, which have severe side-effects.
Pharmaceutical drugs commonly prescribed to combat GI disorders include:
Megestrol acetate (Megace), an anticachectic. This substance can cause high blood pressure, diabetes, inflammation of the blood vessels, congestive heart failure, seizures, and pneumonia. Less serious side effects of this medicine include diarrhea, flatulence, nausea, vomiting, constipation, heartburn, dry mouth, increased salivation, and thrush; impotence, decreased libido, urinary frequency, urinary incontinence, urinary tract infection, vaginal bleeding and discharge; disease of the heart, palpitation, chest pain, chest pressure, and edema; pharyngitis, lung disorders, and rapid breathing; insomnia, headache, weakness, numbness, seizures, depression, and abnormal thinking.
Metronidazole (Flagyl) is carcinogenic. Patients treated with Metronidazole have reported convulsive seizures and peripheral neuropathy. Ironically, this medicine causes problems in the gastrointestinal tract; nausea is reported by about 12% of patients, and is sometimes accompanied by headache, anorexia, and occasionally vomiting; diarrhea; epigastric distress, and abdominal cramping. Constipation has also been reported.
Sulfasalazine (Azulfidine) -Common adverse reactions associated with sulfasalazine are anorexia, headache, nausea, vomiting, gastric distress, and apparently reversible oligospermia. These occur in about one-third of the patients. Less frequent adverse reactions are pruritus, urticaria, fever, Heinz body anemia, hemolytic anemia and cyanosis, which may occur at a frequency of one in every thirty patients or less.
Chlordiazepoxide/Clidinium (Librax) – Drowsiness, ataxia and confusion have been reported in some patients, particularly the elderly and debilitated. Adverse effects reported with use of Librax are those typical of anticholinergic agents, i.e., dryness of the mouth, blurring of vision, urinary hesitancy and constipation. Withdrawal symptoms, similar in character to those noted with barbiturates and alcohol (convulsions, tremor, abdominal and muscle cramps, vomiting and sweating), have occurred following abrupt discontinuance of chlordiazepoxide. Hyoscyamine Sulfate (Levsin) – Adverse reactions may include dryness of the mouth; urinary hesitancy and retention; blurred vision; tachycardia; palpitations; mydriasis; cycloplegia; increased ocular tension; loss of taste; headache; nervousness; drowsiness; weakness; dizziness; insomnia; nausea; vomiting; impotence; suppression of lactation; constipation; bloated feeling; allergic reactions or drug idiosyncrasies; urticaria and other dermal manifestations; ataxia; speech disturbance; some degree of mental confusion and/or excitement (especially in elderly persons); and decreased sweating.
Mesalamine CR (Pentasa) – Common side effects are diarrhea, headache, nausea, abdominal pain, dyspepsia, vomiting, and rash
Phosphorated carbohydrate (Emetrol) – Side effects include: fainting; swelling of face, arms, and legs; unusual bleeding; vomiting; weight loss; yellow eyes or skin. Less common-more common with large doses: Diarrhea; stomach or abdominal pain.
Dicyclomine (Bentyl) – Can cause blurred vision, dry mouth, heart problems, seizures, impotence, and difficulty in urinating, among other effects.
Ciprofloxacin (Cipro) – The most frequent side effects include nausea, vomiting, diarrhea, abdominal pain, rash, headache, and restlessness. Rare allergic reactions have been described, such as hives and anaphylaxis (shock). Methotrexate (Rheumatrex, Trexall) – Is very toxic, depending on dose. The most frequent reactions include mouth sores, stomach upset, and low white blood counts. Methotrexate can cause severe toxicity of the liver and bone marrow, which require regular monitoring with blood testing. It can cause headache and drowsiness, which may resolve if the dose is lowered. Methotrexate can cause itching, skin rash, dizziness, and hair loss.
Diphenoxylate and atropine (Lotomil) – Bad effects include drowsiness, dizziness, and headache, nausea or vomiting, and dry mouth. Euphoria, depression, lethargy, restlessness, numbness of extremities, loss of appetite, and abdominal pain or discomfort has been reported less frequently. Although the dose of atropine in Lomotil is too low to cause side effects when taken in the recommended doses, side effects of atropine (including dryness of the skin and mucous membranes, increased heart rate, urinary retention, and increased body temperature) have been reported, particularly in children under 2.
Prednisone (Delatasone). This is a steroid drug that can have serious adverse musculoskeletal, gastrointestinal, dermatologic, neurological, endocrine, and ophthalmic side effects. These include: congestive heart failure in susceptible patients, potassium loss, hypokalemic alkalosis, sodium retention, and hypertension. Muscle weakness, steroid myopathy, loss of muscle mass, osteoporosis, tendon rupture, vertebral compression fractures, aseptic necrosis of femoral and humeral heads, and pathologic fracture of long bones. Peptic ulcer with possible perforation and hemorrhage; pancreatitis; abdominal distention; ulcerative esophagitis. Impaired wound healing, thin fragile skin, petechiae and ecchymoses, facial erythema. Increased intracranial pressure, usually after treatment, convulsions, vertigo, and headache. Menstrual irregularities; development of Cushingoid state; secondary adrenocortical and pituitary unresponsiveness; decreased carbohydrate tolerance; manifestations of latent diabetes mellitus. Posterior subcapsular cataracts, increased intraocular pressure, glaucoma, and exophthalmos.
Given this verified list of problems that can be created by prescription drugs used to treat gastrointestinal disorders, and the lack of such problems caused by medical marijuana which provides better relief, it's amazing that medical marijuana is not already the most popularly prescribed medication for such disorders.
The overall opinion of enlightened people in the medical community is that medical cannabis can interact with the endogenous cannabinoid system to reduce problems associated with nausea, vomiting, gastric ulcers, irritable bowel syndrome, Crohn's disease, secretory diarrhea, paralytic ileus and gastroesophageal reflux disease.
It is true that cannabis has to be used intelligently. Some people are disconcerted by spiritual, psychological, physiological and emotional changes they experience after using cannabis. Cannabis in rare cases increases schizophrenic symptoms in people who already have a predisposition for schizophrenia. Cannabis can interfere with cognitive function and body coordination, although the impairment is relatively minor and is temporary. Tachycardia and hypotension often occur with cannabis use. Ingesting cannabis as a smoked substance can cause negative effects on the respiratory system. Some people develop a tolerance to cannabis and have to use more and more of it to achieve the same results. Others develop a psychological dependence on it.
As you can see, none of these side effects come close to being as severe as the ones cited for the prescription drugs that cannabis competes with. Licensed medical marijuana grower-patients who grow medical marijuana using organic ingredients and properly engineered synthetic ingredients have told us that cannabis provides a wide range of relief, without severe side-effects, in ways that other drugs, treatment and surgery do not. Medical cannabis patients also report that other interventions are useful during the cannabis regimen. These interventions include detoxifying and cleansing the internal body with herbs, vitamins and supplements, eating only pure, living foods, completely abstaining from junk foods, coffee, caffeine and alcohol, doing yoga and meditation, exercise, nature experiences, and various types of bodywork such as cranial-sacral therapy.

From <http://www.kindgreenbuds.com/medical-marijuana/marijuana-and-treatment-of-digestive-disorders/>

Does Cannabis Help Treat Gastrointestinal Disorders?
OREGON--(ENEWSPF)--July 24, 2012. Below is information about marijuana and gastrointestinal disorders, courtesy of the great people at Americans for Safe Access:
The effectiveness of cannabis for treating symptoms related to gastrointestinal disorders is widely recognized. Its value as an anti-emetic and analgesic has been proven in numerous studies and has been acknowledged by several comprehensive, government-sponsored reviews, including those conducted by the Institute of Medicine (IOM), the U.K. House of Lords Science and Technology Committee, the Australian National Task Force on Cannabis, and others. The IOM concluded, "For patients . . . who suffer simultaneously from severe pain, nausea, and appetite loss, cannabinoid drugs might offer broad-spectrum relief not found in any other single medication."
The most common gastrointestinal disorders-Irritable Bowel Syndrome and Inflammatory Bowel Disease-affect millions of people. The disorders are different, but each causes a great deal of discomfort and distress and both can be disabling. Painful cramping, chronic diarrhea or constipation,

nausea, and inflammation of the intestines are all symptoms of these GI disorders that can be alleviated by cannabis.

Irritable Bowel Syndrome (IBS) is a common disorder of the intestines that leads to stomach pain, gassiness, bloating, constipation, diarrhea or both. Chronic, painful abdominal cramping is common. The cause of IBS is not known, and there is no cure. Researchers have found that the colon muscle of a person with IBS begins to spasm after only mild stimulation. IBS is at least partly a disorder affecting colon motility and sensation.

Inflammatory Bowel Disease (IBD) refers to both Ulcerative Colitis and Crohn's Disease. Ulcerative colitis causes inflammation of the lining of the large intestine, while Crohn's disease causes inflammation of the lining and wall of the large and/or small intestine. The causes of IBD are not known, but there are indications that the disease has a genetic component. The immune system changes that accompany IBD suggest that it may be an immune disorder.

The most common symptoms of Crohn's Disease are pain in the abdomen, diarrhea, and weight loss. There may also be rectal bleeding and fever. The most common complications of Crohn's Disease are blockage of the intestine and ulceration that breaks through into surrounding tissues. Surgery is sometimes required.

The symptoms of Ulcerative Colitis include diarrhea, abdominal cramps, and rectal bleeding. Some people may be very tired and have weight loss, loss of appetite, abdominal pain, and loss of body fluids and nutrients. Joint pain, liver problems, and redness and swelling of the eyes can also occur. Hospitalization and surgery are sometimes needed.

Research on cannabis and GI disorders

Research suggests that cannabis is effective in treating the symptoms of these GI disorders in part because it interacts with the endogenous cannabinoid receptors in the digestive tract, which can result in calming spasms, assuaging pain, and improving motility. Cannabis has also been shown to have anti-inflammatory properties and recent research has demonstrated that cannabinoids are immune system modulators, either enhancing or suppressing immune response.

Cannabis has a long documented history of use in treating GI distress, going back more than a century in western medicine, and far longer in the east. While clinical studies on the use of cannabis for the treatment of gastrointestinal disorders have been largely limited to investigations on nausea suppression and appetite stimulation—two conditions for which cannabis has been consistently shown to be highly effective — the evidence in support of cannabis therapy for other gastrointestinal diseases and disorders is also strong. There is now extensive anecdotal evidence from patients with IBS, Crohn's disease and other painful GI disorders that cannabis eases cramping and helps modulate diarrhea, constipation and acid reflux. Recent laboratory research on the endogenous cannabinoid system in humans has identified that there are many cannabinoid receptors located in both the large and small intestines.

Cannabis and new cannabinoid drugs are attractive for GI treatment because they can address a number of symptoms at once with minimal side effects. Cannabinoids alter how the gut feels, affect the signals the brain sends back and forth to the gut and modulate the actions of the GI tract itself. Beginning in the 1970s, a series of human clinical trials established cannabis' ability to stimulate food intake and weight gain in healthy volunteers. In a randomized trial, THC significantly improved appetite and nausea in comparison with placebo. There were also trends towards improved mood and weight gain. Unwanted effects were generally mild or moderate in intensity. Cannabis helps combat the painful and often debilitating cramping that accompanies many GI disorders because cannabinoids relax contractions of the smooth muscle of the intestines. In fact, smooth-muscle relaxant properties of cannabinoids are so well established that preparations of guinea-pig intestine are routinely used as an in vitro screening tool to test the potency and function of synthetic cannabinoids.

Research on a variety of rodents has shown that endogenous cannabinoids play crucial neuromodulatory roles in controlling the operation of the gastrointestinal system, with synthetic and natural cannabinoids acting powerfully to control gastrointestinal motility and inflammation. Cannabinoid receptors comprise G-protein coupled receptors that are predominantly in enteric and central neurones (CB1R) and immune cells (CB2R). The digestive tract contains endogenous cannabinoids (anandamide and 2-arachidonylglycerol) and cannabinoid CB1 receptors can be found on myenteric and submucosal nerves. Activating cannabinoid receptors has been demonstrated to inhibit gastrointestinal fluid secretion and inflammation in animal models.

In the last decade, evidence obtained from the use of selective agonists and inverse agonists/antagonists indicates that manipulation of CB1R can have significant results. Research has also shown that in the case of intestinal inflammation, the body will increase the number of cannabinoid receptors in the area in an attempt to regulate the inflammation by processing more cannabinoids.

Cannabinoids have a demonstrated ability to block spinal, peripheral and gastrointestinal mechanisms that promote pain in IBS and related disorders. Animal research also indicates that cannabinoids work well in controlling gastroesophageal reflux disease, a condition in which gastric acids attack the esophagus and for which commonly prescribed medications, such as atropine, have serious adverse side effects.

From this evidence, many researchers have concluded that pharmacological modulation of the endogenous cannabinoid system provides new treatment options for a number of gastrointestinal diseases, including nausea and vomiting, gastric ulcers, irritable bowel syndrome, Crohn's disease, secretory diarrhea, paralytic ileus and gastroesophageal reflux disease. The experience of patients with these GI disorders shows that for broad-spectrum relief, cannabis is highly effective and frequently helps when other treatment options prove ineffective.

How Cannabis Compares to Other Treatments

The medications currently employed to fight chronic GI disorders include many that produce serious side effects. These side effects frequently threaten the health of the patient and require other medications to combat them. Drugs commonly prescribed to combat GI disorders include:

Megestrol acetate (Megace), an anticachectic. Serious side effects of this medicine include high blood pressure, diabetes, inflammation of the blood vessels, congestive heart failure, seizures, and pneumonia. Less serious side effects of this medicine include diarrhea, flatulence, nausea, vomiting, constipation, heartburn, dry mouth, increased salivation, and thrush; impotence, decreased libido, urinary frequency, urinary incontinence, urinary tract infection, vaginal bleeding and discharge; disease of the heart, palpitation, chest pain, chest pressure, and edema; pharyngitis, lung disorders, and rapid breathing; insomnia, headache, weakness, numbness, seizures, depression, and abnormal thinking.

Prednisone (Delatasone), like all steroids, can have serious adverse musculoskeletal, gastrointestinal, dermatologic, neurological, endocrine, and ophthalmic side effects. These include: congestive heart failure in susceptible patients, potassium loss, hypokalemic alkalosis, sodium retention, and hypertension. Muscle weakness, steriod myopathy, loss of muscle mass, osteoporosis, tendon rupture, vertebral compression fractures, aseptic necrosis of femoral and humeral heads, and pathologic fracture of long bones. Peptic ulcer with possible perforation and hemorrhage; pancreatitis; abdominal distention; ulcerative esophagitis. Impaired wound healing, thin fragile skin, petechiae and ecchymoses, facial erythema. Increased intracranial pressure (pseudo-tumor cerebri) usually after treatment, convulsions, vertigo, and headache. Menstrual irregularities; development of Cushingoid state; secondary adrenocortical and pituitary unresponsiveness; decreased carbohydrate tolerance; manifestations of latent diabetes mellitus. Posterior subcapsular cataracts, increased intraocular pressure, glaucoma, and exophthalmos.

Metronidazole (Flagyl) has been shown to be carcinogenic in mice and rats. Two serious adverse reactions reported in patients treated with Metronidazole have been convulsive seizures and peripheral neuropathy, the latter characterized mainly by numbness or paresthesia of an extremity. The most common adverse reactions reported have been referable to the gastrointestinal tract, particularly nausea reported by about 12% of patients, sometimes accompanied by headache, anorexia, and occasionally vomiting; diarrhea; epigastric distress, and abdominal cramping. Constipation has been reported.

Sulfasalazine (Azulfidine)—The most common adverse reactions associated with sulfasalazine are anorexia, headache, nausea, vomiting, gastric distress, and apparently reversible oligospermia. These occur in about one-third of the patients. Less frequent adverse reactions are pruritus, urticaria, fever, Heinz body anemia, hemolytic anemia and cyanosis, which may occur at a frequency of one in every thirty patients or less.

Chlordiazepoxide/Clidinium (Librax)—Drowsiness, ataxia and confusion have been reported in some patients, particularly the elderly and debilitated. Adverse effects reported with use of Librax are those typical of anticholinergic agents, i.e., dryness of the mouth, blurring of vision, urinary hesitancy and constipation. Withdrawal symptoms, similar in character to those noted with barbiturates and alcohol (convulsions, tremor, abdominal and muscle cramps, vomiting and sweating), have occurred following abrupt discontinuance of chlordiazepoxide.

Hyoscyamine Sulfate (Levsin)—Adverse reactions may include dryness of the mouth; urinary hesitancy and retention; blurred vision; tachycardia;

palpitations; mydriasis; cycloplegia; increased ocular tension; loss of taste; headache; nervousness; drowsiness; weakness; dizziness; insomnia; nausea; vomiting; impotence; suppression of lactation; constipation; bloated feeling; allergic reactions or drug idiosyncrasies; urticaria and other dermal manifestations; ataxia; speech disturbance; some degree of mental confusion and/or excitement (especially in elderly persons); and decreased sweating.
Mesalamine CR (Pentasa)—The most common side effects are diarrhea, headache, nausea, abdominal pain, dyspepsia, vomiting, and rash.
Phosphorated carbohydrate (Emetrol)—Side effects include: fainting; swelling of face, arms, and legs; unusual bleeding; vomiting; weight loss; yellow eyes or skin. Less common-more common with large doses: Diarrhea; stomach or abdominal pain.
Dicyclomine (Bentyl)—The most common side effects occurring with dicyclomine are due to its anticholinergic activity: dry mouth, blurred vision, confusion, agitation, increased heart rate, heart palpitations, constipation, difficulty urinating, and occasionally seizures can occur. Other potential side effects include changes in taste perception, difficulty swallowing, headache, nervousness, drowsiness, weakness, dizziness, impotence, flushing, difficulty falling asleep, nausea, vomiting, rash, and a bloated feeling.
Ciprofloxacin (Cipro)—The most frequent side effects include nausea, vomiting, diarrhea, abdominal pain, rash, headache, and restlessness. Rare allergic reactions have been described, such as hives and anaphylaxis.
Methotrexate (Rheumatrex, Trexall)—can cause severe toxicity that is usually related to the dose taken. The most frequent reactions include mouth sores, stomach upset, and low white blood counts. Methotrexate can cause severe toxicity of the liver and bone marrow, which require regular monitoring with blood testing. It can cause headache and drowsiness, which may resolve if the dose is lowered. Methotrexate can cause itching, skin rash, dizziness, and hair loss. A dry, non-productive cough can be a result of a rare lung toxicity.
Diphenoxylate and atropine (Lotomil)—The most common side effects include drowsiness, dizziness, and headache, nausea or vomiting, and dry mouth. Euphoria, depression, lethargy, restlessness, numbness of extremities, loss of appetite, and abdominal pain or discomfort have been reported less frequently. Although the dose of atropine in Lomotil is too low to cause side effects when taken in the recommended doses, side effects of atropine (including dryness of the skin and mucous membranes, increased heart rate, urinary retention, and increased body temperature) have been reported, particularly in children under two.
Cannabis—By comparison, the side effects associated with cannabis are typically mild and are classified as “low risk.” Euphoric mood changes are among the most frequent side effects. Cannabinoids can exacerbate schizophrenic psychosis in predisposed persons. Cannabinoids impede cognitive and psychomotor performance, resulting in temporary impairment. Chronic use can lead to the development of tolerance. Tachycardia and hypotension are frequently documented as adverse events in the cardiovascular system. A few cases of myocardial ischemia have been reported in young and previously healthy patients. Inhaling the smoke of cannabis cigarettes induces side effects on the respiratory system. Cannabinoids are contraindicated for patients with a history of cardiac ischemias. In summary, a low risk profile is evident from the literature available. Serious complications are very rare and are not usually reported during the use of cannabinoids for medical indications.
Johnny Green is a marijuana activist from Oregon. He has a Bachelor's Degree in Public Policy. Follow Johnny Green on Facebook, Twitter, Reddit, StumbleUpon, and Digg.
Source: http://www.theweedblog.com

From <http://www.enewspf.com/latest-news/health-and-fitness/34985-marijuana-and-gastrointestinal-disorders.html>

From <http://medicalmarijuana.com/experts/expert/title.cfm?artID=727>

Medical Cannabis News
Gastrointestinal Disorders and Medical Marijuana
Category: Medical Cannabis News
Published: Wednesday, 25 July 2012 01:11
The effectiveness of cannabis for treating symptoms related to gastrointestinal disorders is widely recognized. Its value as an anti-emetic and analgesic has been proven in numerous studies and has been acknowledged by several comprehensive, government-sponsored reviews , including those conducted by the Institute of Medicine (IOM), the U.K. House of Lords Science and Technology Committee, the Australian National Task Force on Cannabis, and others. The IOM concluded, “For patients . . . who suffer simultaneously from severe pain, nausea, and appetite loss, cannabinoid drugs might offer broad-spectrum relief not found in any other single medication.”

The most common gastrointestinal disorders-Irritable Bowel Syndrome and Inflammatory Bowel Disease-affect millions of people. The disorders are different, but each causes a great deal of discomfort and distress and both can be disabling. Painful cramping, chronic diarrhea or constipation, nausea, and inflammation of the intestines are all symptoms of these GI disorders that can be alleviated by cannabis.
Irritable Bowel Syndrome (IBS) is a common disorder of the intestines that leads to stomach pain, gassiness, bloating, constipation, diarrhea or both. Chronic, painful abdominal cramping is common. The cause of IBS is not known, and there is no cure. Researchers have found that the colon muscle of a person with IBS begins to spasm after only mild stimulation. IBS is at least partly a disorder affecting colon motility and sensation.
Inflammatory Bowel Disease (IBD) refers to both Ulcerative Colitis and Crohn’s Disease. Ulcerative colitis causes inflammation of the lining of the large intestine, while Crohn’s disease causes inflammation of the lining and wall of the large and/or small intestine. The causes of IBD are not known, but there are indications that the disease has a genetic component. The immune system changes that accompany IBD suggest that it may be an immune disorder.
The most common symptoms of Crohn’s Disease are pain in the abdomen, diarrhea, and weight loss. There may also be rectal bleeding and fever. The most common complications of Crohn’s Disease are blockage of the intestine and ulceration that breaks through into surrounding tissues. Surgery is sometimes required.
The symptoms of Ulcerative Colitis include diarrhea, abdominal cramps, and rectal bleeding. Some people may be very tired and have weight loss, loss of appetite, abdominal pain, and loss of body fluids and nutrients. Joint pain, liver problems, and redness and swelling of the eyes can also occur. Hospitalization and surgery are sometimes needed.
Research on cannabis and GI disorders
Research suggests that cannabis is effective in treating the symptoms of these GI disorders in part because it interacts with the endogenous cannabinoid receptors in the digestive tract, which can result in calming spasms, assuaging pain, and improving motility. Cannabis has also been shown to have anti-inflammatory properties and recent research has demonstrated that cannabinoids are immune system modulators, either enhancing or suppressing immune response.
Cannabis has a long documented history of use in treating GI distress, going back more than a century in western medicine, and far longer in the east. While clinical studies on the use of cannabis for the treatment of gastrointestinal disorders have been largely limited to investigations on nausea suppression and appetite stimulation—two conditions for which cannabis has been consistently shown to be highly effective — the evidence in support of cannabis therapy for other gastrointestinal diseases and disorders is also strong. There is now extensive anecdotal evidence from patients with IBS, Crohn’s disease and other painful GI disorders that cannabis eases cramping and helps modulate diarrhea, constipation and acid reflux. Recent laboratory research on the endogenous cannabinoid system in humans has identified that there are many cannabinoid receptors located in both the large and small intestines.
Cannabis and new cannabinoid drugs are attractive for GI treatment because they can address a number of symptoms at once with minimal side effects. Cannabinoids alter how the gut feels, affect the signals the brain sends back and forth to the gut and modulate the actions of the GI tract itself. Beginning in the 1970s, a series of human clinical trials established cannabis’ ability to stimulate food intake and weight gain in healthy volunteers. In a randomized trial, THC significantly improved appetite and nausea in comparison with placebo. There were also trends towards improved mood

and weight gain. Unwanted effects were generally mild or moderate in intensity. Cannabis helps combat the painful and often debilitating cramping that accompanies many GI disorders because cannabinoids relax contractions of the smooth muscle of the intestines. In fact, smooth-muscle relaxant properties of cannabinoids are so well established that preparations of guinea-pig intestine are routinely used as an in vitro screening tool to test the potency and function of synthetic cannabinoids.
Research on a variety of rodents has shown that endogenous cannabinoids play crucial neuromodulatory roles in controlling the operation of the gastrointestinal system, with synthetic and natural cannabinoids acting powerfully to control gastrointestinal motility and inflammation. Cannabinoid receptors comprise G-protein coupled receptors that are predominantly in enteric and central neurones (CB1R) and immune cells (CB2R). The digestive tract contains endogenous cannabinoids (anandamide and 2-arachidonylglycerol) and cannabinoid CB1 receptors can be found on myenteric and submucosal nerves. Activating cannabinoid receptors has been demonstrated to inhibit gastrointestinal fluid secretion and inflammation in animal models.
In the last decade, evidence obtained from the use of selective agonists and inverse agonists/antagonists indicates that manipulation of CB1R can have significant results. Research has also shown that in the case of intestinal inflammation, the body will increase the number of cannabinoid receptors in the area in an attempt to regulate the inflammation by processing more cannabinoids.
Cannabinoids have a demonstrated ability to block spinal, peripheral and gastrointestinal mechanisms that promote pain in IBS and related disorders. Animal research also indicates that cannabinoids work well in controlling gastroesophageal reflux disease, a condition in which gastric acids attack the esophagus and for which commonly prescribed medications, such as atropine, have serious adverse side effects.
From this evidence, many researchers have concluded that pharmacological modulation of the endogenous cannabinoid system provides new treatment options for a number of gastrointestinal diseases, including nausea and vomiting, gastric ulcers, irritable bowel syndrome, Crohn's disease, secretory diarrhea, paralytic ileus and gastroesophageal reflux disease. The experience of patients with these GI disorders shows that for broad-spectrum relief, cannabis is highly effective and frequently helps when other treatment options prove ineffective.
Read the full article at theweedblog.com

From <http://cannabisni.com/gastrointestinal-disorders-and-medical-marijuana>
Cannabis Research: gastrointestinal disorders Posted on 15 Jan 13:35
Cannabis Research: gastrointestinal disorders

A gastrointestinal disorder is a disorder in which the bowel appears normal, but is not functioning properly. Common examples of such a disorder are irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and constipation.
Studies in this area:
1) Cannabinoids and the gastrointestinal tract (gastrointestinal tract and the Cannabinoids - 2001)
This study aimed to evaluate the effect of cannabinoids on the digestive system. It was found that the activation of CB1 receptors in the endocannabinoid system inhibits the production of stomach acid, suggesting that it could be used by patients for whom too much is produced and who have irritable bowel symptoms. It has also been found that there appears to be no adverse effects or withdrawal symptoms were, which can be associated with the use of cannabinoids. Published in the journal "Good."
2) Differential expression of cannabinoid receptors in the human colon: Cannabinoids promote epithelial wound healing (Differential Expression of Cannabinoid Receptors in the Human Colon: Cannabinoids Promote Epithelial Wound Healing - 2005)
In this study it was attempted to find the position of the cannabinoid receptors in the large intestine and to determine their function. It was found that the cannabinoid receptors are distributed throughout the colon to find and that they play a role in alleviating IBD. Published in the journal "Gastroenterology."
3) Endocannabinoids and the gastrointestinal tract (gastrointestinal tract and the endocannabinoid - 2006)
This research was designed to explore the historical use of cannabis as a medicine in the treatment of gastrointestinal problems and whether cannabis really has an effect. It was found that the endocannabinoid system serves to protect the Verdauuungssystem against anomalies, which means that cannabis may be an effective way to treat those who suffer from such diseases. Published in the "U.S. National Library of Medicine."
4) Cannabis use in patients with chronic inflammatory bowel disease (Cannabis use amongst patients with inflammatory bowel disease - 2011)
Previous studies suggest that the endocannabinoid system plays a role in preventing and protecting against inflammation in the colon. Through questionnaires, which assessed the habits of cannabis use of patients with related disorders, it was found that the use of cannabis seemed to have a positive therapeutic benefit and further research should be carried out in this direction. Published in the "U.S. National Library of Medicine."

From <http://napavalleyvape.com/blogs/news/16706752-cannabis-research-gastrointestinal-disorders>
Clinical endocannabinoid deficiency (CECD): can this concept explain therapeutic benefits of cannabis in migraine, fibromyalgia, irritable bowel syndrome and other treatment-resistant conditions?
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Abstract
OBJECTIVES:
This study examines the concept of clinical endocannabinoid deficiency (CECD), and the prospect that it could underlie the pathophysiology of migraine, fibromyalgia, irritable bowel syndrome, and other functional conditions alleviated by clinical cannabis.
METHODS:
Available literature was reviewed, and literature searches pursued via the National Library of Medicine database and other resources.
RESULTS:
Migraine has numerous relationships to endocannabinoid function. Anandamide (AEA) potentiates 5-HT1A and inhibits 5-HT2A receptors supporting therapeutic efficacy in acute and preventive migraine treatment. Cannabinoids also demonstrate dopamine-blocking and anti-inflammatory effects. AEA is tonically active in the periaqueductal gray matter, a migraine generator. THC modulates glutamatergic neurotransmission via NMDA receptors. Fibromyalgia is now conceived as a central sensitization state with secondary hyperalgesia. Cannabinoids have similarly demonstrated the ability to block spinal, peripheral and gastrointestinal mechanisms that promote pain in headache, fibromyalgia, IBS and related disorders. The past and potential clinical utility of cannabis-based medicines in their treatment is discussed, as are further suggestions for experimental investigation of CECD via CSF examination and neuro-imaging.
CONCLUSION:
Migraine, fibromyalgia, IBS and related conditions display common clinical, biochemical and pathophysiological patterns that suggest an underlying clinical endocannabinoid deficiency that may be suitably treated with cannabinoid medicines.
Republished from

• Clinical endocannabinoid deficiency (CECD): can this concept explain therapeutic benefits of cannabis in migraine, fibromyalgia, irritable bowel syndrome and other treatment-resistant conditions? [Neuro Endocrinol Lett. 2004]

From <http://www.ncbi.nlm.nih.gov/pubmed/18404144>

Cannabinoid Treatment for Psoriasis Symptoms

Published by Jan

Psoriasis

Psoriasis (/s?'ra?.?s?s/) is a chronic immune-mediated disease that appears on the skin. It occurs when the immune system sends out faulty signals that speed up the growth cycle of skin cells. Psoriasis is not contagious. There are five types of psoriasis: plaque, guttate, inverse, pustular and erythrodermic. The most common form, plaque psoriasis, is commonly seen as red and white hues of scaly patches appearing on the top first layer of the epidermis (skin). Some patients, though, have no dermatological symptoms.
In plaque psoriasis, skin rapidly accumulates at these sites, which gives it a silvery-white appearance. Plaques frequently occur on the skin of the elbows and knees, but can affect any area, including the scalp, palms of hands and soles of feet, and genitals. In contrast to eczema, psoriasis is more likely to be found on the outer side of the joint.
The disorder is a chronic recurring condition that varies in severity from minor localized patches to complete body coverage. Fingernails and toenails are frequently affected (psoriatic nail dystrophy) and can be seen as an isolated symptom. Psoriasis can also cause inflammation of the joints, which is known as psoriatic arthritis. Between ten and forty percent of all people with psoriasis have psoriatic arthritis.
The cause of psoriasis is not fully understood, but it is believed to have a genetic component and local psoriatic changes can be triggered by an injury to the skin known as the Koebner phenomenon . Various environmental factors have been suggested as aggravating to psoriasis, including stress, withdrawal of systemic corticosteroid, as well as other environmental factors, but few have shown statistical significance. There are many treatments available, but because of its chronic recurrent nature, psoriasis is a challenge to treat.

Causes, incidence, and risk factors
Psoriasis is a very common condition. The disorder may affect people of any age, but it most commonly begins between ages 15 and 35. The condition cannot be spread to others. It does seem to be passed down through families. . Doctors think it probably occurs when the body's immune system mistakes healthy cells for dangerous substances
Skin cells grow deep in the skin and normally rise to the surface about once a month. In persons with psoriasis, this process is too fast (about 2 weeks instead of 4 weeks) and dead skin cells build up on the skin's surface.
The following may trigger an attack of psoriasis or make the condition more difficult to treat:
• Bacteria or viral infections, including strep throat and upper respiratory infections
• Dry air or dry skin
• Injury to the skin, including cuts, burns, and insect bites
• Some medicines, including antimalaria drugs, beta-blockers, and lithium
• Stress
• Too little sunlight
• Too much sunlight (sunburn)
• Too much alcohol
In general, psoriasis may be severe in people who have a weakened immune system. This may include persons who have:
• AIDS
• Autoimmune disorders (such as rheumatoid arthritis)
• Cancer chemotherapy
Up to one-third of people with psoriasis may also have arthritis, a condition known as psoriatic arthritis.

Symptoms
The symptoms of psoriasis can manifest in a variety of forms. Variants include plaque, pustular, guttate and flexural psoriasis. This section describes each type
Psoriasis is a chronic relapsing disease of the skin that may be classified into nonpustular and pustular types as follows
Nonpustular
Psoriasis vulgaris (chronic stationary psoriasis, plaque-like psoriasis) (L40.0) is the most common form of psoriasis. It affects 80 to 90% of people with psoriasis. Plaque psoriasis typically appears as raised areas of inflamed skin covered with silvery white scaly skin. These areas are called plaques.
Psoriatic erythroderma (erythrodermic psoriasis) (L40.85) involves the widespread inflammation and exfoliation of the skin over most of the body surface. It may be accompanied by severe itching, swelling and pain. It is often the result of an exacerbation of unstable plaque psoriasis, particularly following the abrupt withdrawal of systemic treatment. This form of psoriasis can be fatal, as the extreme inflammation and exfoliation disrupt the body's ability to regulate temperature and for the skin to perform barrier functioning.
Pustular
Pustular psoriasis appears as raised bumps that are filled with noninfectious pus (pustules). The skin under and surrounding the pustule is red and tender. Pustular psoriasis can be localized, commonly to the hands and feet (palmoplantar pustulosis), or generalized with widespread patches occurring randomly on any part of the body. Types include:
• Generalized pustular psoriasis (pustular psoriasis of von Zumbusch)
• Pustulosis Palmaris et plantaris (persistent palmoplantar pustulosis, pustular psoriasis of the Barber type, pustular psoriasis of the extremities)
• Annular pustular psoriasis
• Acrodermatitis continua
• Impetigo herpetiformis
• Other
Additional types of psoriasis include:
Drug-induced psoriasis
Inverse psoriasis (flexural psoriasis, inverse psoriasis. It appears as smooth inflamed patches of skin. It occurs in skin folds, particularly around the genitals (between the thigh and groin), the armpits, under an overweight abdomen (panniculus), and under the breasts (inframammary fold). It is aggravated by friction and sweat, and is vulnerable to fungal infections.
Napkin psoriasis
Seborrheic-like psoriasis
Guttate psoriasis is characterized by numerous small, scaly, red or pink, teardrop-shaped lesions. These numerous spots of psoriasis appear over large areas of the body, primarily the trunk, but also the limbs and scalp. Guttate psoriasis is often preceded by a streptococcal infection, typically streptococcal pharyngitis. The reverse is not true.
Nail psoriasis produces a variety of changes in the appearance of finger and toenails. These changes include discoloring under the nail plate, pitting of the nails, lines going across the nails, thickening of the skin under the nail, and the loosening (onycholysis) and crumbling of the nail.
Psoriatic arthritis involves joint and connective tissue inflammation. Psoriatic arthritis can affect any joint, but is most common in the joints of the fingers and toes. This can result in a sausage-shaped swelling of the fingers and toes known as dactylitis. Psoriatic arthritis can also affect the hips, knees and spine (spondylitis). About ten to fifteen percent of people who have psoriasis also have psoriatic arthritis.
The migratory stomatitis in the oral cavity mucosa and the geographic tongue that confined to the dorsal and lateral aspects of the tongue mucosa, are believed to be oral manifestations of psoriasis, as being histologically identical to cutaneous psoriasis lesions and more prevalent among psoriasis

patients, although these conditions are quite common in the non-psoriatic population, affecting (1% to 2.5%) of the general population.
Psoriasis is usually graded as mild (affecting less than 3% of the body), moderate (affecting 3–10% of the body) or severe. Several scales exist for measuring the severity of psoriasis. The degree of severity is generally based on the following factors: the proportion of body surface area affected; disease activity (degree of plaque redness, thickness and scaling); response to previous therapies; and the impact of the disease on the person.
Psoriasis can appear suddenly or slowly. In many cases, psoriasis goes away and then flares up again repeatedly.
People with psoriasis have irritated patches of skin. The redness is most often seen on the elbows, knees, and trunk, but it can appear anywhere on the body. For example, there may be flaky patches on the scalp.
The skin patches or dots may be:
• Itchy
• Dry and covered with silver, flaky skin (scales)
• Pink-red in color (like the color of salmon)
• Raised and thick
• Other symptoms may include:
• Genital lesions in males
• Joint pain or aching (psoriatic arthritis)
• Nail changes, including nail thickening, yellow-brown spots, dents (pits) on the nail surface, and separation of the nail from the base
• Severe dandruff on the scalp
Psoriasis may affect any or all parts of the skin. T here are five main types of psoriasis:
1. Erythrodermic -- The skin redness is very intense and covers a large area.
2. Guttate -- Small, pink-red spots appear on the skin.
3. Inverse -- Skin redness and irritation occurs in the armpits, groin, and in between overlapping skin.
4. Plaque -- Thick, red patches of skin are covered by flaky, silver-white scales. This is the most common type of psoriasis.
5. Pustular -- White blisters are surrounded by red, irritated skin.

Treatment
The goal of treatment is to control your symptoms and prevent infections.
In general, three treatment options are used for patients with psoriasis:
1. Topical medications such as lotions, ointments, creams, and shampoos
2. Body-wide (systemic) medications, which are pills or injections that affect the whole body, not just the skin
3. Phototherapy, which uses light to treat psoriasis
Most cases of psoriasis are treated with medications that are placed directly on the skin or scalp:
• Cortisone creams and ointments
• Creams or ointments that contain coal tar or anthralin
• Creams to remove the scaling (usually salicylic acid or lactic acid)
• Dandruff shampoos (over-the-counter or prescription)
• Moisturizers
• Prescription medicines containing vitamin D or vitamin A (retinoids)

If you have an infection, your doctor will prescribe antibiotics.
You may try the following self-care at home:
Oatmeal baths may be soothing and may help to loosen scales. You can use over-the-counter oatmeal bath products. Alternatively, you can mix 1 cup of oatmeal into a tub of warm water.
Sunlight may help your symptoms go away. Be careful not to get sunburned.
Relaxation and antistress techniques may be helpful. The link between stress and flares of psoriasis is not well understood. Some people may choose to have phototherapy.
Phototherapy is a medical treatment in which your skin is carefully exposed to ultraviolet light.
Phototherapy may be given alone or after you take a drug that makes the skin sensitive to light.
Phototherapy for psoriasis can be given as ultraviolet A (UVA) or ultraviolet B (UVB) light.

Systemic Treatment
Persons with very severe psoriasis may receive medicines to suppress the body's immune response. These medicines include methotrexate or cyclosporine. (Persons who have psoriatic arthritis may also receive these drugs.) Retinoids such as acitretin can also be used.
Newer drugs called biologics specifically target the body's immune response, which is thought to play a role in psoriasis. These drugs are used when other treatments do not work. Biologics approved for the treatment of psoriasis include:
1. Adalimumab (Humira)
2. Alefacept (Amevive)
3. Etanercept (Enbrel)
4. Infliximab (Remicade)
5. Stelara

Expectations (prognosis)
Psoriasis is a life-long condition that can be controlled with treatment. It may go away for a long time and then return. With appropriate treatment, it usually does not affect your general physical health.

Complications
• Arthritis
• Pain
• Severe itching
• Secondary skin infections
• Side effects from medicines used to treat psoriasis
• Skin cancer from light therapy

Prevention
There is no known way to prevent psoriasis. Keeping the skin clean and moist and avoiding your specific psoriasis triggers may help reduce the number of flare-ups.
Doctors recommend daily baths or showers for persons with psoriasis. Avoid scrubbing too hard, because this can irritate the skin and trigger an

attack.

Genetics
Psoriasis has a large hereditary component, and many genes are associated with it, but it is not clear how those genes work together. Most of them involve the immune system, particularly the major histocompatibility complex (MHC) and T cells. The main value of genetic studies is they identify molecular mechanisms and pathways. for further study and potential drug targets.
Classic genome wide linkage analysis has identified nine locations (loci) on different chromosomes associated with psoriasis. They are called psoriasis susceptibility 1 through 9 (PSORS1 through PSORS9). Within those loci are genes. Many of those genes are on pathways that lead to inflammation. Certain variations (mutations) of those genes are commonly found in psoriasis.
Genome-wide association scans have identified other genes that are altered to characteristic variants in psoriasis. Some of these genes express inflammatory signal proteins, which affect cells in the immune system that are also involved in psoriasis. Some of these genes are also involved in other autoimmune diseases.
Two major genes under investigation are IL12B on chromosome 5q, which expresses interleukin-12B; and IL23R on chromosome 1p, which expresses the interleukin-23 receptor, and is involved in T cell differentiation. T cells are involved in the inflammatory process that leads to psoriasis. These genes are on the pathway that ends up regulating tumor necrosis factor-α and nuclear factor κB, two genes that are involved in inflammation.

Immunology
In psoriasis, immune cells move from the dermis to the epidermis, where they stimulate skin cells (keratinocytes) to proliferate. Psoriasis does not seem to be a true autoimmune disease. In an autoimmune disease, the immune system confuses an outside antigen with a normal body component, and attacks them both. However, in psoriasis, the inflammation does not seem to be caused by outside antigens (although DNA does have an immunostimulatory effect). Researchers have identified many of the immune cells involved in psoriasis, and the chemical signals they send to each other to coordinate inflammation. At the end of this process, immune cells, such as dendritic cells and T cells, move from the dermis to the epidermis, secreting chemical signals, such as tumor necrosis factor-α, interleukin-1β, and interleukin-6, which cause inflammation, and interleukin-22, which causes keratinocytes to proliferate.
The immune system consists of an innate immune system, and an adaptive immune system.
In the innate system, immune cells have receptors that have evolved to target specific proteins and other antigens that are commonly found on pathogens. In the adaptive immune system, immune cells respond to proteins and other antigens that they may never have seen before, which are presented to them by other cells. The innate system often passes antigens on to the adaptive system. When the immune system makes a mistake, and identifies a healthy part of the body as a foreign antigen, the immune system attacks that protein, as it does in autoimmunity.
In psoriasis, DNA is an inflammatory stimulus. DNA stimulates the receptors on plasmacytoid dendritic cells, which produce interferon-α, an immune stimulatory signal (cytokine). In psoriasis, keratinocytes produce antimicrobial peptides. In response to dendritic cells and T cells, they also produce cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α, which signals more inflammatory cells to arrive and produces further inflammation.
Dendritic cells bridge the innate and adaptive immune system. They are increased in psoriatic lesions, induce the proliferation of T cells, and type 1 helper T cells. Certain dendritic cells can produce tumor necrosis factor-α, which calls more immune cells and stimulates more inflammation. Targeted immunotherapy, and psoralen and ultraviolet A (PUVA) therapy, reduces the number of dendritic cells.
T cells move from the dermis into the epidermis. They are attracted to the epidermis by alpha-1 beta-1 integrin, a signaling molecule on the collagen in the epidermis. Psoriatic T cells secrete interferon-γ and interleukin-17. Interleukin-17 is also associated with interleukin-22. Interleukin-22 induces keratocytes to proliferate. One hypothesis is that psoriasis involves a defect in regulatory T cells, and in the regulatory cytokine interleukin-10

Prognosis
Psoriasis is typically a lifelong condition. There is currently no cure, but various treatments can help to control the symptoms. Many of the most effective agents used to treat severe psoriasis carry an increased risk of significant morbidity including skin cancers, lymphoma and liver disease. However, the majority of people's experience of psoriasis is that of minor localized patches, particularly on the elbows and knees, which can be treated with topical medication. Psoriasis can get worse over time, but it is not possible to predict who will go on to develop extensive psoriasis or those in whom the disease may appear to vanish. Individuals will often experience flares and remissions throughout their lives. Controlling the signs and symptoms typically requires lifelong therapy.

Quality of life
Severe cases of psoriasis has been shown to affect health-related quality of life to an extent similar to the effects of other chronic diseases, such as depression, hypertension, congestive heart failure or type 2 diabetes. Depending on the severity and location of outbreaks, individuals may experience significant physical discomfort and some disability. Itching and pain can interfere with basic functions, such as self-care, walking, and sleep. Plaques on hands and feet can prevent individuals from working at certain occupations, playing some sports, and caring for family members or a home. Plaques on the scalp can be particularly embarrassing, as flaky plaque in the hair can be mistaken for dandruff. Medical care can be costly and time-consuming, and can interfere with an employment or school schedule.
Individuals with psoriasis may also feel self-conscious about their appearance and have a poor self-image that stems from fear of public rejection and psychosexual concerns. Psychological distress can lead to significant depression and social isolation.

Cause
The cause of psoriasis is not fully understood. There are two main hypotheses about the process that occurs in the development of the disease. The first considers psoriasis as primarily a disorder of excessive growth and reproduction of skin cells. The problem is simply seen as a fault of the epidermis and its keratinocytes. The second hypothesis sees the disease as being an immune-mediated disorder in which the excessive reproduction of skin cells is secondary to factors produced by the immune system. T cells (which normally help protect the body against infection) become active, migrate to the dermis and trigger the release of cytokines (tumor necrosis factor-alpha TNFα, in particular) which cause inflammation and the rapid production of skin cells. It is not known what initiates the activation of the T cells.
The immune-mediated model of psoriasis has been supported by the observation that immunosuppressant medications can clear psoriasis plaques. However, the role of the immune system is not fully understood, and it has recently been reported that an animal of psoriasis can be triggered in mice lacking T cells. Animal models, however, reveal only a few aspects resembling human psoriasis.
Psoriasis is a fairly idiosyncratic disease. The majority of people's experience of psoriasis is one in which it may worsen or improve for no apparent reason. . Nevertheless, the first outbreak is sometimes reported following stress (physical and mental), skin injury, and streptococcal infection. Conditions that have been reported as accompanying a worsening of the disease include infections, stress, and changes in season and climate. Certain medicines, including lithium salt, beta-blockers and the antimalarial drug chloroquine have been reported to trigger or aggravate the disease. Excessive alcohol consumption, smoking and obesity may exacerbate psoriasis or make the management of the condition difficult or perhaps these co morbidities are effects rather than causes Hairspray, some face creams and hand lotions, can also cause an outbreak of psoriasis In 1975, Stefania Jablonska and collaborators advanced a new theory that special antibodies tend to break through into the lower layers of the skin and set up a complex series of chemical reactions.
Individuals suffering from the advanced effects of the human immunodeficiency virus, or HIV, often exhibit psoriasis. This presents a paradox to researchers, as traditional therapies that reduce T-cell counts generally cause psoriasis to improve. Yet, as CD4-T-cell counts decrease with the progression of HIV, psoriasis worsens. In addition, HIV is typically characterized by a strong Th2 cytokine profile, whereas psoriasis vulgaris is

characterized by a strong Th1 secretion pattern. The prevalence of psoriasis in the HIV positive population ranges from one to six percent, which is about three times higher than the normal population. Psoriasis in AIDS sufferers is often severe, and untreatable with conventional therapy.
Psoriasis occurs more likely in dry skin than oily or well-moisturized skin, and specifically after an external skin injury such as a scratch or cut (see Koebner phenomenon). This is believed to be caused by an infection, in which the infecting organism thrives under dry skin conditions with minimal skin oil, which otherwise protects skin from infections. The case for psoriasis is opposite to the case of athlete's foot, which occurs because of a fungus infection under wet conditions as opposed to dry in psoriasis. This infection induces inflammation, which causes the symptoms commonly associated with psoriasis, such as itching and rapid skin turnover, and leads to drier skin, as the infecting organism absorbs the moisture that would otherwise go to the skin. To prevent dry skin and reduce psoriasis symptoms, it is advised to not use shower scrubs, as they not only damage skin by leaving tiny scratches, but they also scrape off the naturally occurring skin oil. It is recommended to use talc powder after washing, as that helps absorb excess moisture that would otherwise go to the infecting agent. Additionally, moisturizers can be applied to moisturize the skin, and lotions used to promote skin oil gland functions.

There are a number of different treatment options for psoriasis. Typically, topical agents are used for mild disease, phototherapy for moderate disease, and systemic agents for severe disease
Topical agents
Bath solutions and moisturizers, mineral oil, and petroleum jelly may help soothe affected skin and reduce the dryness that accompanies the build-up of skin on psoriatic plaques. Medicated creams and ointments applied directly to psoriatic plaques can help reduce inflammation, remove built-up scale, reduce skin turn over, and clear affected skin of plaques. Ointment and creams containing coal tar, dithranol (anthralin), corticosteroids like desoximetasone (Topicort), fluocinonide, vitamin D3analogues (for example, calcipotriol), and retinoids are routinely used. The use of the Fingertip unit may be helpful in guiding how much topical treatment to use.[31] The mechanism of action of each is probably different, but they all help to normalize skin cell production and reduce inflammation. Activated vitamin D and its analogues can inhibit skin cell proliferation.
Apricus Biosciences is currently developing PsoriaVa, a topical cream for the treatment of psoriasis. It contains Calcipotriol and Betamethasone as the active ingredients and a permeation enhancer DDAIP that facilitates the delivery of the drug into the blood stream.
Phototherapy
Phototherapy in the form of sunlight has long been used effectively for treatment.[30] Wavelengths of 311–313 nm are most effective and special lamps have been developed for this application.[30] The amount of light used is determined by a person's skin type.[30] Increased rates of cancer from treatment appear to be small.[30]
Psoralen and ultraviolet A phototherapy (PUVA) combines the oral or topical administration of psoralen with exposure to ultraviolet A (UVA) light. The mechanism of action of PUVA is unknown, but probably involves activation of psoralen by UVA light, which inhibits the abnormally rapid production of the cells in psoriatic skin. There are multiple mechanisms of action associated with PUVA, including effects on the skin immune system.
PUVA is associated with nausea, headache, fatigue, burning, and itching. Long-term treatment is associated with squamous cell carcinoma (not with melanoma).
Systemic agents
Psoriasis that is resistant to topical treatment and phototherapy is treated by medications taken internally by pill or injection (systemic). Patients undergoing systemic treatment are required to have regular blood and liver function tests because of the toxicity of the medication. Pregnancy must be avoided for the majority of these treatments. Most people experience a recurrence of psoriasis after systemic treatment is discontinued.
The three main traditional systemic treatments are methotrexate, cyclosporine and retinoids. Methotrexate and cyclosporine are immunosuppressant drugs; retinoids are synthetic forms of vitamin A. Patients taking methotrexate are prone to ulcerations
Biologics are manufactured proteins that interrupt the immune process involved in psoriasis. Unlike generalized immunosuppressant therapies such as methotrexate, biologics focus on specific aspects of the immune function leading to psoriasis. These drugs (interleukin antagonists) are relatively new, and their long-term impact on immune function is unknown, but they have proven effective in treating psoriasis and psoriatic arthritis. Biologics are usually given by self-injection or in a doctor's office. In the United Kingdom in 2005, the British Association of Dermatologists (BAD) published guidelines for use of biological interventions in psoriasis. A UK national register called the BAD Biological Register (BADBIR) has been set up to collect valuable information on side effects (and benefits) and will be used to inform doctors on how best to use biological agents and similar drugs.
In 2008, the FDA approved three new treatment options available to psoriasis patients: 1) Taclonex Scalp, a new topical ointment for treating scalp psoriasis; 2) the Xtrac Velocity excimer laser system, which emits a high-intensity beam of ultraviolet light, can treat moderate to severe psoriasis; and 3) the biologic drug adalimumab (brand name Humira) was also approved to treat moderate to severe psoriasis. Adalimumab had already been approved to treat psoriatic arthritis. The most recent biologic drug that has been approved to treat moderate to severe psoriasis, as of 2010, is ustekinumab (brand name Stelara).
Medications with the least potential for adverse reactions are preferentially employed. If the treatment goal is not achieved, then therapies with greater potential toxicity may be used. Medications with significant toxicity are reserved for severe unresponsive psoriasis. This is called the psoriasis treatment ladder. As a first step, medicated ointments or creams, called topical treatments, are applied to the skin. If topical treatment fails to achieve the desired goal, then the next step would be to expose the skin to ultraviolet (UV) radiation. This type of treatment is called phototherapy. The third step involves the use of medications that are taken internally by pill or injection. This approach is called systemic treatment.
Alternative therapy
Some studies suggest psoriasis symptoms can be relieved by changes in diet and lifestyle. Fasting periods, low energy diets and vegetarian diets have improved psoriasis symptoms in some studies, and diets supplemented with fish oil (in this study cod liver oil) have shown beneficial effects. Fish oils are rich in the two omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and contain Vitamin E, furthermore cod liver oil contains Vitamin A and Vitamin D.
The severity of psoriasis symptoms may also be influenced by lifestyle habits related to alcohol, smoking, weight, sleep, stress and exercise.
Another treatment is ichthyotherapy, which is practiced at some spas in Turkey, Croatia, Ireland, Hungary and Serbia. Doctor fish living in outdoor thermal pools are encouraged to feed on the psoriatic skin, only consuming the affected areas. The outdoor location of the spa may also have a beneficial effect. This treatment can provide temporary relief of symptoms. A revisit to the spas every few months is often required. This treatment has been examined in two small clinical trials, with positive results.
Hypnotherapy may be effective for psoriasis.
A psychological symptom management program has been reported as being a helpful adjunct to traditional therapies in the management of psoriasis.
It has been suggested that cannabis might treat psoriasis, due to the anti-inflammatory properties of its cannabinoids, and the regulatory effects of THC on the immune system.
On 17 November 2008, researchers led by Yin-Ku Lin of Chang Gung Memorial Hospital and Chang Gung University in Taiyuan, Taiwan, told Reuters by telephone that Indigo naturalis (Qing dai, ??), a dark blue plant used in traditional Chinese medicine, appears to be effective in treating psoriasis. In the latest issue of Archives of Dermatology, they wrote, "The Indigo naturalis ointment-treated lesions showed an eighty one percent improvement over placebo.

Cannabinoids as Treatment for Psoriasis Symptoms
Research:
cannabinoids inhibit keratinocyte proliferation, and therefore support a potential role for cannabinoids in the treatment of psoriasis.
Cannabis might treat psoriasis, due to the anti-inflammatory properties of its cannabinoids, and the regulatory effects of THC on the immune system.
The adverse effects of cannabis might be overcome by use of more specific cannabinoid receptor medications, to inhibit keratinocyte proliferation.

Moreover, they observe that cannabinoids are readily absorbed through the skin. This is the ideal method of treating psoriasis, as it avoids some of the toxicity associated with systemic therapies.
The researchers tested the effects of four plant cannabinoids – Δ-9 tetrahydrocannabinol, cannabidiol, cannabinol and cannabigerol – on rapidly proliferating, cultured human keratinocytes.
All four cannabinoids inhibited keratinocyte growth in a dose-dependent manner, they report.
Despite varying degrees of affinity for cannabinoid receptors (CBs) among the substances tested, the extent of growth inhibition was similar with all four cannabinoids, implying a non-specific effect.
In confirmation of this, the investigators found that selective CB2 agonists only partially inhibited keratinocyte growth, while a non-selective CB agonist had a concentration-dependent effect.
Neither CB1 nor CB2 antagonists attenuated the effects of the CB agonists or the cannabinoids, however. Furthermore, these antagonists actually exhibited direct dose-dependent inhibition of keratinocyte growth.
Cannabinoids, as novel additions to the antipsoriatic ammunition.
Source
Dermatology Department, Shiraz University of Medical Sciences, Shiraz, Iran. namazi_mr@yahoo.com
cannabinoids, which exert inhibitory effects on antigen processing and macrophage/T-cell interaction and on the release of IL-2, TNF-alpha and nitric oxide from immune cells.
Testimonials:
Cannabinoids extracted with grape seed oil: can be taken orally or topically applied or both.
“Smoking marijuana keeps me from having flare-ups of psoriasis.”
““HempEase” made from cannabis roots (no THC) has powerful skin treating qualities”.
Cannabinoids can be taken orally, and applied topically for the treatment of psoriasis symptoms with little to no side effects.

Sativa x Indica hybrid

Side effects of psoriasis medication s (pharmaceuticals) include:
? Severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue); black, tarry stools; blood in the urine; change in the appearance of a mole; chest pain; confusion; dark urine; diarrhea; fast or irregular heartbeat; flushing of the face, chest, back, or abdomen; gum disease or overgrowth; increased or decreased urination; loss of coordination; mental or mood changes; muscle cramps; numbness or tingling of the skin; seizures; severe or persistent headache or dizziness; shortness of breath; symptoms of infection (eg, chills, cough, fever, painful urination, sore throat); tremors; unusual bleeding or bruising; unusual lumps; unusual thickening or growth on the skin; unusual tiredness or weakness; vision changes; wheezing; yellowing of the skin or eyes, acne, dizziness, headache, increased hair growth, runny nose, sleeplessness, vomiting and stomach discomfort.

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Writing in the Journal of Dermatological Science, Jonathan Wilkinson (Nottingham University) and Elizabeth Williamson (Reading University) say that cannabinoids have anti-inflammatory properties and can inhibit the growth of cancer cell lines.

From <http://medicalmarijuana.com/medical-marijuana-treatments/Psoriasis>

Ozonated organic hemp seed oil
The benefits ozonated olive oil and hemp seed oil
Skip to content

Fungal infections
Powered by

Translate

Severe fungal-infection – bottom of foot

Nail Fungus Infection
Fungal infections – athletes foot, nail bed – (contagious)
Although athlete’s foot and nail fungus are two different types of fungal infections, they are contagious.
Do not share towel or shoes with those who has fungal infections.
Athletes foot advice
Athlete’s Foot keep clean and dry, wearing shoes that are made of breathable material, such as leather.
Another option is wearing an airy shoe like sandals or perforated shoes. These types of shoes will help to keep the feet from sweating.
Athletes foot symptoms:
Bad foot odor
Itching / burning sensation
Peeling / cracking of skin
Itchy
Blisters

Bleeding
Pain
Cellulitis
Thick, crumbling and discolored nails
Nail fungus symptoms: – Nail fungus is a fungal infection that often occurs at your finger and/or toenails
Thickened nails
Nails that become brittle, crumbly, or ragged
Nails that seem to be distorted in shape
Nails that seem to have become flat or dull, having lost luster and shine
White spots on the nail
Discolored nails: yellow, green, or black in color. Caused by debris building up under the nail.
Nail separation from nail bed
Natural Remedy – Ozonated hemp oil
1) Wash your foot or infected area with mild soap and rinse thoroughly in warm / cold water (your choice), then pat dry with clean towel.
2) Spray or drop the ozonated hemp oil onto your feet or toes.
3) Gently rub the oil all over the infected area .
4) Apply at least 2 to 3 times a day – Morning, noon and night.
5)For better quicker healing process , please wear perforated or airy shoes.
Time to recover depends on how severe your fungal infection condition. For light fungal infection only takes 2- 3 days to completely heal.

Home Remedy with Ozonated hemp seed oil for bacterial infection skin diseases
Go to products page

From <http://www.oilozonated.com/fungal-infections>
Hemp Root – A Little Known Cure for Muscle Pain

As if this plant couldn't do everything – there is yet another application.

Hemp-Eaze Products
About a month ago I pulled the muscles in my lower back. I was bed ridden for about 2 days with severe muscle swelling. It hurt so bad that I could barely walk.
I would ice it to ease the swelling but nothing really helped. Ibuprofin, ice, tiger balm. It only helped a little.
Then Hemp to the rescue.
I googled Hemp and Back pain as I laid in bed and found a little known, but highly effective product called Hemp-Eaze. I will admit I was hesitant. Although I'm a big proponant of hemp, I had never heard of the root being useful.
But according to Darcy Stoddard the creator of Hemp-Eaze:
The proprietary blend of Hemp-Eaze rejuvenates circulation, promotes cellular growth and encourages deep tissue healing. It relieves sore muscles and bone aches by reducing swelling and relieving pain which enables the healing of sprains, arthritic joints, and joint pain. The choice of herbs for Hemp-Eaze is designed to help eliminate bruising and mend wounds by reducing damaged blood vessels.
And I am a testament to the healing properties of this product. Within a couple hours the swelling in my back went down enough for me to walk around – Two more days and I was up and running agin. Now I use Hemp-eaze on sore muscles after lifting at the gym and have given it to a friend who suffers from arthritis. It seems to be working for her as well.
According to the Hemp-Eaze website
Medicinal use of Hemp Root originated in Central Asia and was cultivated in China as early as 2,800 B.C. The first recordings attributing to the healing powers of Hemp root was by Chinese pharmacopoeia. Around 2,000 B.C., Ancient Egypt gave the world one of it's first medical texts, The Ebers Papyrus. It contains some 800 recipes and refers to hemp root remedies several times. In Medieval Europe physicians prescribed the root to alleviate the agonies of gout and other painful diseases. The root was also made into an oiled salve for burns. Warmed oiled root paste was massaged into sore muscles and used for arthritis, easing swelling and painful joints. Among the vast array of useful native American plants is hemp. Listed for it's ability to ease pain reduce swelling and soothe a variety of disorders. Although the root has no THC,it has locked in the healing elements, of cannasativine, that have proven to be a great anti-inflammatory painkilling agent with deep penetration
So try it for yourself… Hemp-Eaze. A great product made from an incredible plant.

Other stories you might be interested in:
33. New Product: Hemp Shield Deck Finish
34. Medical Marijuana opening doors for Industrial Hemp production
35. Hemp ain't for Hippies anymore – New Technologies On The Rise
36. UPDATE!! California Senator is Poised to Welcome Hemp

From <http://www.hemp.com/2011/02/hemp-root-a-little-known-cure-for-muscle-pain/>

Cannabinoids as antioxidants and neuroprotectants
CA 2329626 A1
Abstract
Cannabinoids have been found to have antioxidant properties, unrelated to NMDA
receptor antagonism. This new found property makes the cannabinoids useful in the treatment and prophylaxis of wide variety of oxidation associated diseases, such as ischemic, age-related, inflammatory and autoimmune diseases.
The cannabinoids are found to have particular application as neuroprotectants, for example in limiting neurological damage following ischemic insults, such as stroke and trauma, or in the treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and HIV dementia. Nonpsychoactive cannabinoids, such as cannabidoil, are particularly advantageous to use because they avoid toxicity that is encountered with psychoactive cannabinoids at high doses useful in the method of the present invention. A particular disclosed class of cannabinoids useful as neuroprotective antioxidants is formula (I) wherein the R group is independently selected from the group consisting of H, CH3, and COCH3.
Claims(35)
1. A method of treating diseases caused by oxidative stress, comprising administering a therapeutically effective amount of a cannabinoid to a subject who has a disease caused by oxidative stress.
2. The method of claim 1, wherein the cannabinoid is nonpsychoactive.
3. The method of claim 2, wherein the cannabinoid has a volume of distribution of 10 L/kg or more.
4. The method of claim 2, wherein the cannabinoid is not as agonist at the NMDA
and AMPA receptors.
5. The method of claim 1, wherein the cannabinoid is Image not available. View PDF where R is selected from the group of H, substituted or

unsubstituted alkyl, carboxyl, alkoxy, aryl, aryloxy, arylalkyl, halo or amino.
6. The method of claim 5, wherein R is selected from the group consisting of H, and substituted or unsubstituted alkyl, or carboxyl, alkoxy.
7. The method of claim 2, wherein the cannabinoid is selected from the group Image not available. View PDF where A is selected from the group of cyclohexyl and substituted or unsubstituted aryl, Image not available. View PDF but not a pinene R1 is selected from the group of H, substituted or unsubstituted alkyl, and substituted or unsubstituted carboxyl;
R2 is selected from the group of H, lower substituted or unsubstituted alkyl, and alkoxy;
R3 is selected from the group of H, lower substituted or unsubstituted alkyl, and substituted or unsubstituted carboxyl;
R4 is selected from the group of H, hydroxyl and lower substituted or unsubstituted alkyl; and R5 is selected from the group of H, hydroxyl and lower substituted or unsubstituted alkyl;
8. The method of claim 7, wherein R1 is selected from the group of lower alkyl, COOH and COCH3;
R2 is selected from the group of unsubstituted C1-C5 alkyl, hydroxyl, methoxy and ethoxy;
R3 is selected from the group of H, unsubstituted C1-C3 alkyl, and COCH3;
R4 is selected from the group of hydroxyl, pentyl, heptyl and diemthylheptyl;
R5 is selected from the group of hydroxyl and methyl;
9. The method of claim 1, wherein the cannabinoid is selected from the group of Image not available. View PDF wherein R1, R2 and R3 are independently selected from the group consisting of H, CH3, and COCH3.
10. The method of claim 9, wherein the cannabinoid is selected from the group of:

Image not available. View PDF where: a) R1=R2=R3=H
b) R1=R3=H, R2=CH3 c) R1=R2=CH3, R3=H
d) R1=R2=COCH3, R3=H
e) R1=H, R2=R3=OCH3
11. The method of claim 2, wherein the cannabinoid is selected from the group of:

Image not available. View PDF wherein R19 is H, lower alkyl, lower alcohol, or carboxyl; R20 is H or OH; and R21-R25 are independently H or OH.
12. The method of claim 11, wherein R19 is H, CH3, CH2OH, or COOH, and R20-R25 are independently H or OH.
13. The method of claim 2, wherein the cannabinoid is Image not available. View PDF wherein R19 and R20 are H, and R26 is alkyl.
14. The method of claim 10, wherein the cannabinoid is cannabidiol.
15. A method of treating ischemic or neurodegenerative disease in the central nervous system of a subject, comprising administering to the subject a therapeutically effective amount of a cannabinoid of claim 5.
16. The method of claim 13, wherein the cannabinoid is not a psychoactive caanabinoid.
17. The method of claim 15, wherein the ischemic or neurodegenerative disease is selected from the group consisting of an ischemic infarct, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus dementia, Down's syndrome, and heart disease.
18. An assay for selecting a cannabinoid to use in treating a neurological disease, comprising determining whether the cannabinoid is as antioxidant.
19. A method of treating a neurological or ischemic disease, comprising using the assay of claim 18 to determine whether the cannabinoid is an antioxidant, and administering the cannabinoid if it is as antioxidant
20. The method of claim 19, wherein the neurological or ischemic disease is a vascular ischemic event in the central nervous system or myocardium.
21. The method of claim 20, wherein the neurological disease is a thromboembolism in the brain.
22. A method of treating a disease with a cannabinoid, comprising determining whether the disease is caused by oxidative stress, and if the disease is caused by oxidative stress, administering the cannabinoid in a therapeutically effective antioxidant amount
23. The method of claim 22, wherein the cannabinoid has a volume of distribution of at least 1.5 L/kg and substantially no activity at the cannabinoid receptor.
24. The method of claim 23, wherein the cannabinoid has a volume of distribution of at least 10 L/kg.
25. The method of claim 1, wherein the cannabinoid selectively inhibits an enzyme activity of 5- and 15-lipoxygenase more than an enzyme activity of 12-lipoxygenase.
26. An assay for selecting a cannabinoid to use in treating a neurological disease, comprising determining whether the cannabinoid selectively inhibits an enzyme activity of 5- and 15-lipoxygenase substantially more than the enzyme activity of an 12-lipoxygenase.
27. A method of treating a neurological or ischemic disease, comprising using the assay of claim 26 to determine whether the cannabinoid inhibits the enzyme activity of 5- and 15-lipoxygenase substantially more than 12-lipoxygenase, and administering the cannabinoid if it inhibits the enzyme activity of 5- and 15-lipoxygenase substantially more than 12-lipoxygenase.
28. The method of claim 27, wherein the neurological or ischemic disease is a vascular ischemic event in the central nervous system or myocardium:
29. The method of claim 28, wherein the neurological disease is a thromboembolism in the brain.
30. The method of claim 27, wherein the cannabinoid is administered during and after an ischemic injury.
31. The assay of claim 18 further comprising determining whether the cannabinoid selectively inhibits an enzyme activity of 5- and 15-lipoxygenase substantially more than the enzyme activity of an 12-lipoxygenase, and determining whether the cannabinoid has low NMDA antagonist activity.
32. The method of claim 31, further comprising determining whether the cannabinoid possess potent cannabinoid receptor activity.
33. Any of the compounds in claims 9-13, for use in the treatment of a neurodegenerative disease in the central nervous system of a subject
34. Cannabidiol for use in the treatment of an ischemic or neurodegenerative disease in the central nervous system of a subject.
35. The use of claim 34, wherein the ischemic or neurodegenerative disease is selected from the group consisting of an ischemic infarct, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus dementia, Down's syndrome, and heart disease.
35. The use of claim 35 wherein the disease is an ischemic infarct 37. The use of cannabidiol for the manufacture of a medicament for the treatment of a neurodegenerative disease.
38. The use of cannabidiol as in claim 37, wherein the neurodegenerative disease is an ischemic disease of the brain.
Description (OCR text may contain errors)
CANNABINOIDS AS ANTIOXIDANTS AND NEUROPROTECTANTS
FIELD OF THE INVENTION
The present invention concerns pharmaceutical compounds and compositions that are useful as tissue protectants, such as neuroprotectants and cardioprotectants. The compounds and compositions may be used, for example, in the treatment of acute ischemic neurological insults or chronic neurodegenerative diseases.
BACKGROUND OF THE INVENTION
Permanent injury to the central nervous system (CNS) occurs in a variety of medical conditions, and has been the subject of intense scientific scrutiny in recent years. It is known that the brain has high metabolic requirements, and that it can suffer permanent neurologic damage if deprived of sufficient oxygen (hypoxia) for even a few minutes. In the absence of oxygen (anoxia), mitochondrial production of ATP cannot meet the metabolic requirements of the brain, and tissue damage occurs. This process is exacerbated by neuronal release of the neurotransmitter glutamate, which stimulates NMDA (N-methyl-D-aspartate), AMPA
(a-amino-3-hydroxy-5-methyl-4-isoxazole propionate) and kainate receptors. Activation of these receptors initiates calcium influx into the neurons, and production of reactive oxygen species, which are potent toxins that damage important cellular structures such as membranes, DNA
and enzymes.

The brain has many redundant blood supplies, which means that its tissue is seldom completely deprived of oxygen, even during acute ischemic events caused by thromboembolic events or trauma. A combination of the injury of hypoxia with the added insult of glutamate toxicity is therefore believed to be ultimately responsible for cellular death. Hence if the additive insult of glutamate toxicity can be alleviated, neurological damage could also be lessened. Anti-oxidants and anti-inflammatory agents have been proposed to reduce damage, but they often have poor access to structures such as the brain (which are protected by the blood brain barrier).

Given the importance of the NMDA, AMPA and kainate receptors in the mechanism of injury, research efforts have focused on using antagonists to these receptors to interfere with the receptor mediated calcium influx that ultimately leads to cellular death and tissue necrosis. In vitro studies using cultured neurons have demonstrated that glutamate receptor antagonists reduce neurotozicity, but NMDA and AMPA/kainate receptor antagonists have different effects. Antagonists to NMDAr prevent neurotoxicity if present during the glutamate exposure period, but are less effective if added after glutamate is removed. In contrast, AMPA/kainate receptor antagonists are not as effective as NMDA antagonists during the glutamate exposure period, but are more effective following glutamate exposure.

Some of the research on these antagonists has focused on cannabinoids, a subset of which have been found to be NMDA receptor antagonists. U.S. Patent No.
5,538,993 (3S,4S-delta-6-tetrahydrocannabinol-7-oic acids), U.S. Patent No. 5,521,215 (sterospecific (+) THC
enantiomers), and U.S. Patent No. 5,284,867 (dimethylheptyl benzopyrans) have reported that these cannabinoids are effective NMDA receptor blockers. U.S. Patent No.
5,434,295 discloses that the 1,1 dimethylheptyl (DMH) homolog of [3R,4RJ-7-hydroxy-06T'HC (known as HU-210) is a superpotent cannabinoid receptor agonist with cannabinomimetic activity two orders of magnitude greater than the natural O9 THC. The HU-210 dimethyIhepryl cannabinoid, has severe side effects, including fatigue, thirst, headache, and hypotension. J. Pharmacol. Sci.
60:1433-1457 (1971).

Subjects who received this synthetic cannabinoid with a dimethylheptyl group experienced marked psychomotor retardation, and were unwilling or incapable of assuming an erect position.

In contrast to HU-210, the (-)(3R,4R) THC-DMH enantiomer (known as HU-211) displays low affinity to the cannabinoid receptors, but retains NMDA receptor antagonist neuroprotective activity.

Me Me THC (tetrahydrocannabinol) is another of the cannabinoids that has been shown to be neuroprotective in cell cultures, but this protection was believed to be mediated by interaction at the cannabinoid receptor, and so would be accompanied by undesired psychotropic side effects.

CH2~3CH3 THC

Although it has been unclear whether cannabimimetic activity plays a role in neuroprotection against glutamate induced neurological injury, the teaching in this field has clearly been that a cannabinoid must at least be an antagonist at the NMDA receptor to have neuroprotective effect. Hence cannabidiol (2-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl)-5-pentyl-1,3-benzenediol or CBD), a cannabinoid devoid of psychoactive effect (Pharm. Rev. 38:21-43, 1986), has not been considered useful as a neuroprotectant. Cannabidiol has been studied as an antiepileptic (Carlini et al., J. Clin. Pharmacol. 21:417S-4275, 1981; Karler et al., J. Clin.
Plzarmacol. 21:4375-448S, 1981, Consroe et al., J. Clin Pharmacol. 21:428S-4365, 1981), and has been found to lower intraocular pressure (Colasanti et al, Exp. Eye Res.
39:251-259, 1984 and Gen. Pharmac. 15:479-484, 1984).

H2C=

Cannabidiol (CBD) CH2~3C~"~3 No signs of toxicity or serious side effects have been observed following chronic administration of cannabidiol to healthy volunteers (Cunha et al., Pharmacology 21:175-185, 1980), even in large acute doses of 700 mg/day (Consroe et al., Pharmacol.
Biochem. Behav.
40:701-708, 1991) but cannabidiol is inactive at the NMDA receptor. Hence in spite of its potential use in treating glaucoma and seizures, cannabidiol has not been considered a neuroprotective agent that could be used to prevent glutamate induced damage in the central nervous system.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a new class of antioxidant drugs, that have particular application as neuroprotectants, although they are generally useful in the treatment of many oxidation associated diseases.

Yet another object of the invention is to provide a subset of such drugs that can be substantially free of psychoactive or psychotoxic effects, are substantially non-toxic even at very high doses, and have good tissue penetration, for example crossing the blood brain barrier.

It has surprisingly been found that cannabidiol and other cannabinoids can function as neuroprotectants, even though they lack NMDA receptor antagonist activity.

This discovery was made possible because of the inventor's recognition of a previously unanticipated antioxidant property of the cannabinoids in general (and cannabidiol in particular) that functions completely independently of antagonism at the NMDA, AMPA and kainate receptors. Hence the present invention includes methods of preventing or treating diseases caused by oxidative stress, such as neuronal hypoxia, by administering a prophylactic or therapeutically effective amount of a cannabinoid to a subject who has a disease caused by oxidative stress.

The cannabinoid may be a cannabinoid other than THC, HU-210, or other potent cannabinoid receptor agonists. The cannabinoid may also be other than HU-211 or any other NMDA receptor antagonist that has previously been reported. A potent cannabinoid receptor agonist is one that has an ECso at the cannabinoid receptor of 50 nM or less, but in more particular embodiments 190 nM or 250 nM or less. In disclosed embodiments the cannabinoid is not psychoactive, and is not psychotoxic even at high doses. In some particularly disclosed embodiments, the cannabinoid is selected from the group:

Ra where A is aryl, and particularly but not a pinene such as:
and the R,-RS groups are each independently selected from the groups of hydrogen, lower substituted or unsubstituted alkyl, substituted or unsubstituted carboxyl, substituted or unsubstituted alkoxy, substituted or unsubstituted alcohol, and substituted or unsubstituted ethers, and R~ - R~ are H or methyl. In particular embodiments, there are no rutrogens in the rings, and/or no amino substitutions on the rings.

In other embodiments, the cannabinoid is one of the following:
Ris Rl \ I Rg R
Rt2 B I R1 s R~ 1 R9 R9 Rlo Rlo R~ R1 Rts A ~ R~
Rg R1 R8 Rt2 R ' C I
1~ Rls ~ R

Rto where there can be 0 to 3 double bonds on the A ring,~as indicated by the optional double bonds indicated by dashed lines on the A ring. The C ring is aromatic, and the B
ring can be a pyran.

Particular embodiments are dibenzo pyrans and cyclohexenyl benzenediols.

Particular embodiments of the cannabinoids of the present invention may also be highly lipid soluble, and in particular embodiments can be dissolved in an aqueous solution only sparingly (for example 10 mg/ml or less). The actanol/water partition ratio at neutral pH in useful embodiments is 5000 or greater, for example 6000 or greater. This high lipid solubility enhances penetration of the drug into the CNS, as reflected by its volume of distribution (Vd) of 1.5 L/kg or more, for example 3.5 L/kg, 7 L/kg, or ideally 10 L/kg or more, for example at least 20 L/kg.

Particular embodiments may also be highly water soluble derivatives that are able to penetrate the CNS, for example carboxyl derivatives.

R,_,8 are independently selected from the group of H, substituted or unsubstituted alkyl, especially lower alkyl, for example unsubstituted C,-C3 alkyl, hydroxyl, alkoxy, especially lower alkoxy such as methoxy or ethoxy, substituted or unsubstituted alcohol, and unsubstituted or substituted carboxyl, for example COOH or COCH3. In other embodiments R,_,8 can also be substituted or unsubstituted amino, and halogen.

WO 99/53917 PCT/US99l08769 The cannabinoid has substantially no binding to the NMDAr (for example an ICso greater than or equal to 5 ~M or 10 p.M), has substantially no psychoactive activity mediated by the cannabinoid receptor (for example an IC~o at the cannabinoid receptor of greater than or equal to 300 nM, for example greater than 1 p.M and a K; greater than 250 nM, especially 500-1000 nM, for example greater than 1000 nM), and antioxidant activity, as demonstratable by the Fenton reaction or cyclic voltametry.
In other particular embodiments, the cannabinoids are one of the following:
Ri9 \ Rz~ R2o H

\
/ OH

where R,9 is substituted or unsubstituted alkyl, such as lower alkyl (for example methyl), lower alcohol (such as methyl alcohol) or carboxyl (such as carboxylic acid) and oxygen (as in =O); RZO is hydrogen or hydroxy; R2, is hydrogen, hydroxy, or methoxy; RZZ is hydrogen or hydroxy; R23 is hydrogen or hydroxy; R24 is hydrogen or hydroxy; RZS is hydrogen or hydroxy;
and RZ6 is substituted or unsubstituted alkyl (for example n-methyl alkyl), substituted or unsubstituted alcohol, or substituted or unsubstituted carboxy.
In yet other embodiments of the invention, the cannabinoids are 2' 1' ~ R29 3' 4' ORZg s' CSH1 t H3C to wherein numbering conventions for each of the ring positions are shown, and R2,, R28 and R29 are independently selected from the group consisting of H, unsubstituted lower alkyl such as CH3, and carboxyl such as COCH3. Particular examples of nonpsychoactive cannabinoids that fall within this definition are cannabidiol and Rzz Rza _7_ 'H

2' ~~ny'H~

6' \ ~ 4' O C
CH3 still and other structural analogs of cannabidiol.
In more particular embodiments, the cannabinoid is used to prevent or treat an ischemic or neurodegenerative disease in the centrai nervous system of a subject, by administering to the subject a therapeutically effective amount of a cannabinoid to protect against oxidative injury to the central nervous system. The cannabinoid may be any of the compounds set forth above, or more specifically ... I ~ /R29 ., - 3.
6 I / 4.

OR2g s' CsHll H3CIo wherein Rz.,, Rza and Rz9 are independently selected from the group consisting of H, lower alkyl such as CH3, and carboxyl such as COCH3, and particularly wherein a) Rz., = Rz8 = Rz9 = H
b) Rz~ = Rz9 = H; Rz8 = CH3 c) Rz~ = Rza = CH3; Rz9 = H
d) Rz, = Rz8 = COCH3; Rz9 = H
e) Rz~ = H; Rz8 = Rz9 = COCH~
When Rz,=Rz8=Rz9=H, then the compound is cannabidiol. When Rz,=Rz9=H and Rz8=CH3, the compound is CBD monomethyl ether. When Rz~=Rz8=CH3 and Rz9=H, the compound is CBD
dimethyl ether. When Rz~=Rze=COCH3 and Rz9=H, the compound is CBD diacetate.
When Rz,=H and Rze=Rz9=COCH3, the compound is CBD monoacetate. The ischemic or neurodegenerative disease may be, for example, an ischemic infarct, Alzheimer's disease, _g_ Parkinson's disease, Down's syndrome, human immunodeficiency virus (HIV) dementia, myocardial infarction, or treatment and prevention of intraoperative or perioperative hypoxic insults that can leave persistent neurological deficits following open heart surgery requiring heart/lung bypass machines, such as coronary artery bypass grafts (CABG).
The invention also includes an assay for selecting a cannabinoid to use in treating a neurological disease by determining whether the cannabinoid is an antioxidant.
Once it has been determined that the cannabinoid is an antioxidant, an antioxidant effective amount of the cannabinoid is administered to treat the neurological disease, such as a vascular ischemic event in the central nervous system, for example the type caused by a neurovascular thromboembolism.
Similarly, the method of the present invention includes determining whether a disease is caused by oxidative stress, and if the disease is caused by oxidative stress, administering the cannabinoid in a therapeutically effective antioxidant amount.
The invention also includes identifying and administering antioxidant and neuroprotective compounds (such as cannabidiol) which selectively inhibit the enzyme activity of both 5- and 15-lipoxygenase more than the enzyme activity of 12-lipoxygenase.
In addition, such compounds posses low NMDA antagonist activity and low cannabinoid receptor activity. Assays for selecting compounds with the desired effect on lipoxygenase enzymes, and methods for using identified compounds to treat neurological or ischemic diseases are also provided.
Such diseases may include a vascular ischemic event in the central nervous system, for example a thromboembolism in the brain, or a vascular ischemic event in the myocardium.
Useful administration of the compounds involves administration both during and after an ischemic injury.
These and other objects of the invention will be understood more clearly by reference to the following detailed description and drawings.
BRIEF DESCRIPTION OF THE FIGURES
FIG. lA is a graph showing NMDA induced cellular damage in a neuron (as measured by LDH release) in cells that were exposed to glutamate for 10 minutes, which demonstrates that increasing concentrations of cannabidiol in the cell culture protects against cellular damage.
FIG. 1B is a graph similar to FIG. lA, but showing that AMPA/kainate receptor mediated damage (induced by glutamate and the AMPA/kainate receptor potentiating agents cyclothiazide or concanavalin A) is also reduced in a concentration dependent manner by the presence of cannabidiol in the culture medium.
FIG. 2A is a bar graph showing cellular damage (as measured by LDH release) in the presence of glutamate alone (100 uM Glu), and in the presence of glutamate and 5 pM
cannabidiol (CBD) or 5 p,M THC, and demonstrates that CBD and THC were similarly protective.

_g_ FIG. 2B is a bar graph similar to FIG. 2A, but showing the cellular damage assessed in the presence of the cannabinoid receptor antagonist SR 141716A
(SR), which was not found to alter the neuroprotective effect of CBD (5 ~M) or THC (5 ~,M), indicating the effect is not a typical cannabinoid effect mediated by the cannabinoid receptor.
FIG. 3 is a graph showing the reduction oxidation potentials determined by cyclic voltametry for some natural and synthetic cannabinoids, the antioxidant BHT, and the non-cannabinoid anandamide (arachidonyl ethanolamide) which is a ligand for the cannabinoid receptor.
The voltage at which initial peaks occur is an indication of antioxidant activity.
FIG. 4 is a graph that demonstrates the antioxidant properties of BHT, CBD and THC, by plotting the fluorescence of a fluorescent dye against concentrations of these substances, where declining fluorescence is an indication of greater antioxidant activity.

FIG. 5A is a graph illustrating decreased t-butyl peroxide induced toxicity (as measured by LDH release) in the presence of increasing concentrations of cannabidiol, demonstrating that cannabidiol is an effective antioxidant in living cells.
FIG. 5B is a bar graph comparing the antioxidant activity of several antioxidants against glutamate induced toxicity in neurons, showing that CBD has superior antioxidant activity.
FIG. 6A is a graph showing the effect of CBD (as measured by the change in absorbance at 234 nm) on the enzymatic activity of two lipoxygenase enzymes, rabbit 15-LO and porcine 12-LO, which demonstrates that CBD inhibits 15-LO, but not 12-LO
enzyme.
' 20 FIG. 6B is a graph demonstrating that inhibitory effect of CBD on 15-LO
is competitive.
FIG. 7A is a graph similar to FIG. 6A, but was performed in whole cells rather than purified enzyme preparations, and shows the effect of CBD {as measured by the change in absorbance at 236 nm) on the enzymatic activity of 5-LO from cultured rat basophillic leukemia cells (RBL-2H3), which demonstrates that CBD inhibits 5-LO.
FIG. 7B is a graph showing the effect of CBD (as measured by the change in absorbance at 236 nm) on the formation of 12-HETE (the product of 12-LO) by human leukocytes (12-LO typel).
FIG. 7C is a graph similar to FIG. 7B, showing the effect of CBD (as measured by the change in absorbance at 236 nm) on the formation of 12-HETE by human platelets (12-LO type 2).
FIG. 8 is a bar graph demonstrating that 12-HETE can protect cortical neurons from NMDAr toxicity most effectively when administered during and post ischemia.

This invention provides antioxidant compounds and compositions, such as pharmaceutical compositions, that include cannabinoids that act as free radical scavengers for use in prophylaxis and treatment of disease. The invention also includes methods for using the antioxidants in prevention and treatment of pathological conditions such as ischemia (tissue hypoxia), and in subjects who have been exposed to oxidant inducing agents such as cancer chemotherapy, toxins, radiation, or other sources of oxidative stress. The compositions and methods described herein are also used for preventing oxidative damage in transplanted organs, for inhibiting reoxygenation injury following reperfusion of ischemic tissues (for example in heart disease), and for any other condition that is mediated by oxidative or free radical mechanisms of injury. In particular embodiments of the invention, the compounds and compositions are used in the treatment of ischemic cardiovascular and neurovascular conditions, and neurodegenerative diseases. However the present invention can also be used as an antioxidant treatment in non-neurological diseases.
Molecular oxygen is essential for aerobic organisms, where it participates in many biochemical reactions, including its role as the terminal electron acceptor in oxidative phosphorylation. However excessive concentrations of various forms of reactive oxygen species and other free radicals can have serious adverse biological consequences, including the peroxidation of membrane lipids, hydroxylation of nucleic acid bases, and the oxidation of sulthydryl groups and other protein moieties. Biological antioxidants include tocopherols and tocotrieneols, carotenoids, quinones, bilirubin, ascorbic acid, uric acid, and metal binding proteins.
However these endogenous antioxidant systems are often overwhelmed by pathological processes that allow permanent oxidative damage to occur to tissue.
Free radicals are atoms, ions or molecules that contain an unpaired electron, are usually unstable, and exhibit short half lives. Reactive oxygen species (ROS) is a collective term, designating the oxygen radicals (e.g. 'Oz superoxide radical), which by sequential univalent reduction produces hydrogen peroxide (HZOZ) and hydroxyl radical (' OH). The hydroxyl radical sets off chain reactions and can interact wish nucleic acids. Other ROS include nitric oxide (NO') and peroxy nitrite (NOO'), and other peroxyl (R02') and alkoxyl (RO') radicals. Increased production of these poisonous metabolites in certain pathological conditions is believed to cause cellular damage through the action of the highly reactive molecules on proteins, lipids and DNA.
In particular, ROS are believed to accumulate when tissues are subjected to ischemia, particularly when followed by reperfusion.
The pharmaceutical compositions of the present invention have potent antioxidant and/or free radical scavenging properties, that prevent or reduce oxidative damage in biological systems, such as occurs in ischemic/reperfusion injury, or in chronic neurodegenerative diseases such as Alzheimer's disease, HIV dementia, and many other oxidation associated diseases.

DEFINITIONS
"Oxidative associated diseases" refers to pathological conditions that result at least in part from the production of or exposure to free radicals, particularly oxyradicals, or reactive oxygen species. It is evident to those of skill in the art that most pathological conditions are multifactorial, and that assigning or identifying the predominant causal factors for any particular condition is frequently difficult. For these reasons, the term "free radical associated disease"
encompasses pathological states that are recognized as conditions in which free radicals or ROS
contribute to the pathology of the disease, or wherein administration of a free radical inhibitor (e.g.
desferrozamine), scavenger (e.g. tocopherol, glutathione) or catalyst (e.g.
superoxide dismutase, catalase) is shown to produce detectable benefit by decreasing symptoms, increasing survival, or providing other detectable clinical benefits in treating or preventing the pathological state.
Oxidative associated diseases include, without limitation, free radical associated diseases, such as ischemia, ischemic reperfusion injury, inflammatory diseases, systemic lupus erythematosis, myocardial ischemia or infarction, cerebrovascular accidents (such as a thromboembolic or hemorrhagic stroke) that can lead to ischemia or an infarct in the brain, operative ischemia, traumatic hemorrhage (for example a hypovolemic stroke that can lead to CNS
hypoxia or anoxia), spinal cord trauma, Down's syndrome, Crohn's disease, autoimmune diseases (e.g. rheumatoid arthritis or diabetes), cataract formation, uveitis, emphysema, gastric ulcers, oxygen toxicity, neoplasia, undesired cellular apoptosis, radiation sickness, and others. The present invention is believed to be particularly beneficial in the treatment of oxidative associated diseases of the CNS, because of the ability of the cannabinoids to cross the blood brain barrier and exert their antioxidant effects in the brain. In particular embodiments, the pharmaceutical composition of the present invention is used for preventing, arresting, or treating neurological damage in Parkinson's disease, Alzheimer's disease and HIV dementia; autoimmune neurodegeneration of the type that can occur in encephalitis, and hypoxic or anoxic neuronal damage that can result from apnea, respiratory arrest or cardiac arrest, and anoxia caused by drowning, brain surgery or trauma (such as concussion or spinal cord shock).
As used herein, an "antioxidant" is a substance that, when present in a mixture containing an ozidizable substrate biological molecule, significantly delays or prevents oxidation of the substrate biological molecule. Antioxidants can act by scavenging biologically important reactive free radicals or other reactive oxygen species (' OZ , H20z, ' OH, HOCI, ferryl, peroxyl, peroxynitrite, and alkoxyl), or by preventing their formation, or by catalytically converting the free radical or other reactive oxygen species to a less reactive species. Relative antioxidant activity can be measured by cyclic voltametry studies of the type disclosed in Example S
(and FIG. 3), where the voltage (x-axis) is an index of relative antioxidant activity. The voltage at which the first peak occurs is an indication of the voltage at which an electron is donated, which in turn is an index of antioxidant activity.

"Therapeutically effective antioxidant doses" can be determined by various methods, including generating an empirical dose-response curve, predicting potency and efficacy of a congener by using quantitative structure activity relationships (QSAR) methods or molecular modeling, and other methods used in the pharmaceutical sciences. Since oxidative damage is generally cumulative, there is no minimum threshold level (or dose) with respect to efficacy.
However, minimum doses for producing a detectable therapeutic or prophylactic effect for particular disease states can be established.

As used herein, a "cannabinoid" is a chemical compound (such as cannabinol, THC
or cannabidiol) that is found in the plant species Cannabis sativa (marijuana), and metabolites and synthetic analogues thereof that may or may not have psychoactive properties.
Cannabinoids therefore include (without limitation) compounds (such as THC) that have high affinity for the cannabinoid receptor (for example K; < 250 nM), and compounds that do not have significant affinity for the cannabinoid receptor (such as cannabidiol, CBD). Cannabinoids also include compounds that have a characteristic dibenzopyran ring structure (of the type seen in THC) and cannabinoids which do not possess a pyran ring (such as cannabidiol). Hence a partial list of cannabinoids includes THC, CBD, dimethyl heptylpentyl cannabidiol (DMHP-CBD), 6,12-dihydro-6-hydroxy-cannabidiol (described in U.S. Patent No. 5,227,537, incorporated by reference);
(3S,4R)-7-hydroxy-0~-tetrahydrocannabinol homologs and derivatives described in U.S. Patent No.
4,876,276, incorporated by reference; (+)-4-(4-DMH-2,6-diacetoxy-phenyl]-2-carboxy-6,6-dimethylbicyclo[3.1.1]hept-2-en, and other ~l-phenylpinene derivatives disclosed in U.S. Patent No.
5,434,295, which is incorporated by reference; and cannabidiol (-)(CBD) analogs such as (-)CBD-monomethylether, (-)CBD dimethyl ether; (-)CBD diacetate; (-)3'-acetyl-CBD
monoacetate; and tAFl i, all of which are disclosed in Consroe et al., J. Clin. Pharmacol.
21:4285-436S, 1981, which is also incorporated by reference. Many other cannabinoids are similarly disclosed in Agurell et al., Pharmacol. Rev. 38:31-43, 1986, which is also incorporated by reference.
As referred to herein, the term "psychoactivity° means "cannabinoid receptor mediated psychoactivity." Such effects include, euphoria, lightheadedness, reduced motor coordination, and memory impairment. Psychoactivity is not meant to include non-cannabinoid receptor mediated effects such as the anxiolytic effect of CBD.
The "lipoxygenase enzyme activity" refers to the relative level of lipoxygenase enzyme activity for a particular lipoxgenase, such as 5-, 15- or 12-lipoxygenase, as measured in Example 8. A compound would be said to "selectively inhibit a lipoxgenase enzyme" if the concentration of inhibitor required to reduce enzyme activity by 50% was at least about 5 times less than the amount required to reduce activity of a second lipoxgenase enzyme by the same degree (under the same conditions, i.e. temperature, substrate concentration, etc.) An "antagonist" is a compound that binds and occupies a receptor without activating it. In the presence of a sufficient concentration of antagonist, an agonist cannot activate WO 99/53917 PCT/US99/0$769 its receptor. Therefore, antagonists may decrease the neurotoxicity mediated by NMDA (as described in Example 3) or AMPA and Kainate (as described in Example 4).
An "agonist" is a compound that activates a receptor. When the receptor is activated for a longer than normal period of time, this may cause neurotoxicity, as in the case of NMDA, AMPA and kainate receptors (see Examples 3 and 4).
The term "alkyl" refers to a cyclic, branched, or straight chain alkyl group containing only carbon and hydrogen, and unless otherwise mentioned contains one to twelve carbon atoms. This term is further exemplified by groups such as methyl, ethyl, n-propyl, isobutyl, t-butyl, pentyl, pivalyl, heptyl, adamantyl, and cyclopentyl. Alkyl groups can either be unsubstituted or substituted with one or more substituents, e.g. halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-I-yl, piperazin-1-yl, or other functionality.
The term "lower alkyl" refers to a cyclic, branched or straight chain monovalent alkyl radical of one to seven carbon atoms. This term is further exemplified by such radicals as methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl (or 2-methyipropyl), cyclopropylmethyl, i-amyl, n-amyl, hexyl and heptyl. Lower alkyl groups can also be unsubstituted or substituted, where a specific example of a substituted alkyl is I ,1-dimethyl heptyl.
"Hydroxyl" refers to -OH.
'20 "Alcohol" refers to R-OH, wherein R is alkyl, especially lower alkyl (for example in methyl, ethyl or propyl alcohol). An alcohol may be either linear or branched, such as isopropyl alcohol.
"Carboxyl" refers to the radical -COOH, and substituted carboxyl refers to -COR
where R is alkyl, lower alkyl or a carboxylic acid or ester.
The term "aryl" or "Ar" refers to a monovalent unsaturated aromatic carbocyclic group having a single ring (e.g. phenyl) or multiple condensed rings (e.g.
naphthyl or anthryl), which can optionally be tmsubstituted or substituted with, e.g., halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-I-yl, piperazin-1-yl, or other functionality.
The term "alkoxy" refers to a substituted or ttnsubstituted alkoxy, where an alkoxy has the structure -O-R, where R is substituted or unsubstituted alkyl. In an unsubstituted alkoxy, the R is an unsubstituted alkyl. The term "substituted alkoxy" refers to a group having the structure -O-R, where R is alkyl which is substituted with a non-interfering substituent. The term "arylalkoxy" refers to a group having the structure -O-R-Ar, where R is alkyl and Ar is an aromatic substituent. Arylalkoxys are a subset of substituted alkoxys.
Examples of useful substituted alkoxy groups are: benzyloxy, naphthyloxy, and chlorobenzyloxy.

The term "aryloxy" refers to a group having the structure -O-Ar, where Ar is an aromatic group. A particular aryloxy group is phenoxy.
The term "heterocycle" refers to a monovalent saturated, unsaturated, or aromatic carbocyclic group having a single ring (e.g. morpholino, pyridyl or furyl) or multiple condensed rings (e.g. indolizinyl or benzo[b]thienyl) and having at least one heteroatom, defined as N, O, P, or S, within the ring, which can optionally be unsubstituted or substituted with, e.g. halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.
"Arylalkyl° refers to the groups -R-Ar and -R-HetAr, where Ar is an aryl group.
HetAr is a heteroaryl group, and R is a straight-chain or branched chain aliphatic group. Example of arylaklyl groups include benzyl and furfuryl. Arylalkyl groups can optionally be unsubstituted or substituted with, e.g., halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, peperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.
The term "halo° or "halide" refers to fluoro, bromo, chloro and iodo substituents.
The term "amino" refers to a chemical functionality -NR'R" where R' and R" are independently hydrogen, alkyl, or aryl. The term "quaternary amine" refers to the positively charged group -N+R'R", where R'R" and R" are independently selected and are alkyl or aryl. A
particular amino group is -NHi.
A "pharmaceutical agent" or "drug" refers to a chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.
All chemical compounds include both the (+) and (-) stereoisomers, as well as either the (+) or (-) stereoisomer.
Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (1985} and The Condensed Chemical Dictionary (1981).
The following examples show that both nonpsychoactive cannabidiol, and psychoactive cannabinoids such as THC, can protect neurons from glutamate induced death, by a mechanism independent of cannabinoid receptors. Cannabinoids are also be shown to be potent antioxidants capable of preventing ROS toxicity in neurons.

Preparation of Cannabinoids and Neuronal Cultures Cannabidiol, THC and reactants other than those specifically listed below were purchased from Sigma Chemical, Co. (St. Louis, MO). Cyclothiazide, glutamatergic ligands and MK-801 were obtained from Tocris Cookson (UK).
Dihydrorhodamine was supplied by Molecular Probes (Eugene, OR). T-butyl hydroperoxide, tetraethylammonium chloride, ferric citrate and sodium

dithionite were all purchased from Aldrich (WI). All culture media were Gibco/BRL (MD) products.
S Solutions of cannabinoids, cyclothiazide and other lipophiles were prepared by evaporating a 10 mM ethanolic solution (under a stream of nitrogen) in a siliconized microcentrifuge tube. Dimethyl sulfoxide (DMSO, less than 0.05 % of final volume) was added to ethanol to prevent the lipophile completely drying onto the tube wall. After evaporation, 1 ml of culture media was added and the drug was dispersed using a high power sonic probe. Special attention was used to ensure the solution did not overheat or generate foam.
Following dispersal, all solutions were made up to their final volume in siliconized glass tubes by mixing with an appropriate quantity of culture media.
Primary neuronal cultures were prepared according to the method of Ventra et al.
(J. Neurochem. 66:1752-1761, 1996). Fetuses were extracted by Cesarian section from a 17 day pregnant Wistar rat, and the fetal brains were placed into phosphate buffered saline. The cortices were then dissected out, cut into small pieces and incubated with papain for nine minutes at 37°C. After this time the tissue was dissociated by passage through a fire polished Pasteur pipette, and the resultant cell suspension separated by centrifugation over a gradient consisting of 10 mg/ml bovine serum albumin and 10 mg/ml ovomucoid (a trypsin inhibitor) in Earls buffered salt solution. The pellet was then re-suspended in high glucose, phenol red free Dulbeco's modified Eagles medium containing 10% fetal bovine serum, 2 mM glutamine, 100 IU penicillin, and 100 ~g/ml streptomycin (DMEM). Cells were counted, tested for vitality using the trypan blue exclusion test and seeded onto poly-D-lysine coated 24 multiwell plates. After 96 hours, 10 ~.M fluoro-deoxyuridine and 10 ~M uridine were added to block glial cell growth. This protocol resulted in a highly neuron-enriched culture.

Preparation of Astrocytes and Conditioned Media Astrocyte conditioned DMEM was used throughout the AMPA/kainate toxicity procedure and following glutamate exposure in the NMDAr mediated toxicity protocol. Media was conditioned by 24 hour treatment over a confluent layer of type I astrocytes, prepared from two day old Wistar rat pups. Cortices were dissected, cut into smail pieces, and enzymatically digested with 0.25 % trypsin. Tissue was then dissociated by passage through a fire polished Pasteur pipette and the cell suspension plated into untreated 75 cmz T-flasks. After 24 hours the media was replaced and unattached cells removed. Once astrocytes achieved confluence, cells were divided into four flasks. Media for experiments was conditioned by a 24 hour exposure to these astrocytes, after which time it was frozen at -20°C until use. Astrocyte cultures were used to condition DMEM for no longer than two months.

NMDA Mediated Toxicity Studies Glutamate neurotoxicity can be mediated by NMDA, AMPA or kainate receptors.
To examine NMDAr mediated toxicity, cultured neurons (cultured for 14-18 days) were exposed to 250 p.M glutamate for 10 minutes in a magnesium free saline solution. The saline was composed of 125 mM NaCI, 25 mM glucose, 10 mM HEPES (pH 7.4), 5 mM KC1, 1.8 mM calcium chloride and 5 % bovine serum albumin. Following exposure, cells were washed twice with saline, and incubated for 18 hours in conditioned DMEM. The level of lactate dehydrogenase (LDH) in the media was used as an index of cell injury.
Toxicity was completely prevented by addition of the NMDAr antagonist, MK-801 (500 nM, data not shown). However, FIG. lA shows that cannabidiol also prevented neurotoxicity (maximum protection 88 t 9%) with an ECs° of 2-4 pM (specifically about 3.5 p,M).

AMPA and Kainate Receptor Mediated Toxicity Studies Unlike NMDA receptors, which are regulated by magnesium ions, AMPA/kainate receptors rapidly desensitize following ligand binding. To examine AMPA and kainate receptor mediated toxicity, neurons were cultured for 7-13 days, then exposed to 100 p.M glutamate and 50 pM cyclothiazide (used to prevent AMPA receptor desensitization). Cells were incubated with glutamate in the presence of 500 nM MK-801 (an NMDAr antagonist) for 18-20 hours prior to analysis. Specific AMPA and kainate receptor ligands were also used to separately examine the effects of cannabinoids on AMPA and kainate receptor mediated events.
Fluorowillardiine (1.5 pM) was the AMPA agonist and 4-methyl glutamate (10 pM) was the kainate agonist used to investigate receptor mediated toxicity. When specifically examining kainate receptor activity, cyclothiazide was replaced with 0.15 mg/ml Concanavalin-A.
Cannabidiol protection against AMPA/kainate mediated neurotoxicity is illustrated in FIG. 1B, where LDH in the media was used as an index of cell injury. The neuroprotective effect of cannabidiol was similar to that observed in the NMDA mediated toxicity model (FIG.
lA). Cannabidiol prevented neurotoxicity (maximum protection 80 ~ 17%) with an ECso of 2-4 pM (specifically about 3.3 ~,M). Comparable results were obtained with either the AMPA receptor ligand, fluorowillardiine or the kainate receptor specific ligand, 4-methyl-glutamate (data not shown). Hence cannabidiol protects similarly against toxicity mediated by NMDA, AMPA or kainate receptors.

Unlike cannabidiol, THC is a ligand (and agonist) for the brain cannabinoid receptor. The action of THC at the cannabinoid receptor has been proposed to explain the ability of THC to protect neurons from NMDAr toxicity in vitro. However in AMPA/kainate receptor toxicity assays, THC and cannabidiol were similarly protective (FIG. ZA), indicating that S cannabinoid neuroprotection is independent of cannabinoid receptor activation. This was confirmed by inclusion of cannabinoid receptor antagonist SR-141716A in the culture media (SR in FIG. 2B).
See Mansbach et al., Psychopharmacology 124:315-22, 1996, for a description of SR-141716A.
Neither THC nor cannabidiol neuroprotection was affected by cannabinoid receptor antagonist (FIG. 2B).

Cyclic Voltametery Studies of ReDox Potentials To investigate whether cannabinoids protect neurons against glutamate damage by reacting with ROS, the antioxidant properties of cannabidiol and other cannabinoids were assessed.
Cyclic voltametry, a procedure that measures the ability of a compound to accept or donate electrons under a variable voltage potential, was used to measure the oxidation potentials of several natural and synthetic cannabinoids. These studies were performed with an EG&G
Princeton Applied Research potentiostat/galvanostat (Model 273/ PAR 270 software, NJ).
The working electrode was a glassy carbon disk with a platinum counter electrode and silver/silver chloride ' 20 reference. Tetraethylammonium chloride in acetonitrile (0.1 M) was used as an electrolyte. Cyclic voltametry scans were done from + 0 to 1.8 V at scan rate of 100 mV per second. The reducing ability of cannabidiol (CBD), THC, HU-211, and BHT were measured in this fashion.
Anandamide, a cannabinoid receptor ligand without a cannabinoid like structure, was used as a non-responsive control. Each experiment was repeated twice with essentially the same results.
Cannabidiol, THC and the synthetic cannabinoid HU-211 all donated electrons at a similar potential as the antioxidant BHT. Anandamide (arachidonyl ethanolamide) did not undergo oxidation at these potentials (FIG. 3). Several other natural and synthetic cannabinoids, including cannabidiol, nabilone, and levanantrodol were also tested, and they too exhibited oxidation profiles similar to cannabidiol and THC (data not shown).

Iron Catalyzed Dihydrorhodamine Oxidation (Fenton Reaction) The ability of cannabinoids to be readily oxidized, as illustrated in Example 5, indicated they possess antioxidant properties comparable to BHT. The antioxidant activity of BHT
was examined in a Fenton reaction, in which iron is catalyzed to produce ROS.
Cannabidiol (CBD) and tetrahydrocannabinol (THC) were evaluated for their ability to prevent oxidation of dihydrorhodamine to the fluorescent compound rhodamine. Oxidant was generated by ferrous catalysis (diothionite reduced ferric citrate) of t-butyl hydroperoxide in a 50:50 water:acetonitrile (v/v) solution. Dihydrorhodamine (50 pM) was incubated with 300 pM t-butyl hydroperoxide and 0.5 pM iron for 5 minutes. After this time, oxidation was assessed by spectrofluorimetry (Excit=500 nm, Emiss=570 nm). Various concentrations of cannabinoids and BHT
were included S to examine their ability to prevent dihydrorhodiamine oxidation.
Cannabidiol, THC and BHT all prevented dihydrorhodamine oxidation in a similar, concentration dependent manner (FIG. 4), indicating that cannabinoids have antioxidant potency comparable to BHT.
To confirm that cannabinoids act as antioxidants in the intact cell, neurons were also incubated with the oxidant t-butyl hydroperoxide and varying concentrations of cannabidiol (FIG. SA). The t-butyl hydroperoxide oxidant was chosen for its solubility in both aqueous and organic solvents, which

facilitates oxidation in both cytosolic and membrane cell compartments.
Cell toxicity was assessed 18-20 hours after insult by measuring lactate dehydrogenase (LDH) release into the culture media. All experiments were conducted with triple or quadruple values at IS each point and all plates contained positive (glutamate alone) and baseline controls. The assay was validated by comparison with an XTT based metabolic activity assay. As shown in FIG. SA, cannabidiol protected neurons against ROS toxicity in a dose related manner, with an ECSO of about 6 ~,M. The maximum protection observed was 88 ~ 9%.
Cannabidiol was also compared with known antioxidants in an AMPA/kainate toxicity protocol. Neurons were exposed to 100 ~,M glutamate and equimolar (5 p,M) cannabidiol, a-tocopherol, BHT or ascorbate (FIG. SB). Although all of the antioxidants attenuated glutamate toxicity, cannabidiol was significantly more protective than either a-tocopherol or ascorbate. The similar antioxidant abilities of cannabidiol and BHT in this chemical system (FIG. 4), and their comparable protection in neuronal cultures (FIG. SB), implies that cannabidiol neuroprotection is due to an antioxidant effect.

In vivo Rat Studies The middle cerebral artery of chloral hydrate anesthetized rats was occluded by insertion of suture thread into it. The animals were allowed to recover from the anesthetic and move freely for a period of two hours. After this time the suture was removed under mild anesthetic and the animals allowed to recover for 48 hours. Then the animals were tested for neurological deficits, sacrificed, and the infarct volume calculated. To examine the infarct volume, animals were anesthetized, ex-sanguinated, and a metabolically active dye (3-phenyl tetrazolium chloride) was pumped throughout the body. All living tissues were stained pink by the dye, while morbid regions of infarcted tissue remained white. Brains were then fined for 24 hours in formaldehyde, sliced and the infarct volumes measured.

One hour prior to induction of ischemia 20 mg/kg of catmabidiol was administered by infra-peritoneal injection (ip) in a 90% saline : 5 % emulphor 620 (emulsifier) : 5 % ethanol vehicle. A second ip 10 mg/kg dose of cannabidiol was administered 8 hours later using the same vehicle. Control animals received injections of vehicle without drug. IV doses would be expected to be 3-5 times less because of reduction of first pass metabolism. The infarct size and neurological assessment of the test animals is shown Table 1.
Table 1: Cannabidiol protects rat brains from ischemia damage Volume of Infarct (mm3) Behavioral Deficit Score Animal Drug Control Drug Control 1 108.2 110.5 3 2 2 83.85 119.6 4 4 3 8.41 118.9 3 4 4 75.5 177.7 1 4 5 60.53 33.89 1 3 6 27.52 255.5 1 5 7 23.16 143 1 4 Mean 55.3 137.0 2.0 3.7 SEM 13.8 25.7 0.5 0.4 p=0.OI6 significant p=0.015 significant *Neurological scoring is performed on a subjective 1-5 scale of impairment. 0 = no impairment, 5 = severe (paralysis) This data shows that infarct size was approximately halved in the animals treated with cannabidiol, which was also accompanied by a substantial improvement in the neurological status of the animal.
These studies with the nonpsychotropic marijuana constituent, cannabidiol, demonstrate that protection can be achieved against both glutamate neurotoxicity and free radical induced cell death. THC, the psychoactive principle of cannabis, also blocked glutamate neurotozicity with a potency similar to cannabidiol. In both cases, neuroprotection is unaffected by the presence of a cannabinoid receptor antagonist. These results therefore surprisingly demonstrate that cannabinoids can have useful therapeutic effects that are not mediated by cannabinoid receptors, and therefore are not necessarily accompanied by psychoactive side effects. Cannabidiol also acts as an anti-epileptic and anxiolytic, which makes it particularly useful in the treatment of neurological diseases in which neuroanatomic defects can predispose to seizures (e.g. subarachnoid hemorrhage).
A particular advantage of the cannabinoid compounds of the present invention is that they are highly iipophilic, and have good penetration into the central nervous system. The volume of distribution of some of these compounds is at least 100 L in a 70 kg person {1.4 L/kg), more particularly at least 250 L, and most particularly 500 L or even 700 L in a 70 kg person (10 L/kg). The lipophilicity of particular compounds is also about as great as that of THC, cannabidiol or other compounds that have excellent penetration into the brain and other portions of the CNS.
Cannabinoids that lack psychoactivity or psychotoxicity are particularly useful embodiments of the present invention, because the absence of such side effects allows very high doses of the drug to be used without encountering unpleasant side effects (such as dysphoria) or dangerous complications (such as obtundation in a patient who may already have an altered mental status). For example, therapeutic antioxidant blood levels of cannabidiol can be 5-20 mg/kg, without significant toxicity, while blood levels of psychoactive cannabinoids at this level would produce obtundation, headache, conjunctiva) irritation, and other problems.
Particular examples of the compounds of the present invention have low affinity to the cannabinoid receptor, for example a K; of greater than 250 nM, for example K; >_ 500-1000 nM. A compound with a K;
>_ 1000 nM is particularly useful, which compound has essentially no psychoactivity mediated by the cannabinoid receptor.
Cannabidiol blocks glutamate toxicity with equal potency regardless of whether the insult is mediated by NMDA, AMPA or kainate receptors.
Cannabidiol and THC
have been shown to be comparable to the antioxidant BHT, both in their ability to prevent dihydrorhodamine oxidation and in their cyclic voltametric profiles. Several synthetic cannabinoids also exhibited profiles similar to the BHT, although anandamide, which is not structurally related to cannabinoids, did not. These findings indicate that cannabinoids act as antioxidants in a non-biological situation;
which was confirmed in living cells by showing that cannabidiol attenuates hydroperoxide induced neurotoxicity. The potency of cannabidiol as an antioxidant was examined by comparing it on an equimolar basis with three other commonly used compounds.
In the AMPA/kainate receptor dependent neurotoxicity model, cannabidiol neuroprotection was comparable to the potent antioxidant, BHT, but significantly greater than that observed with either a-tocopherol or ascorbate. This unexpected superior antioxidant activity (in the absence of BHT tumor promoting activity) shows for the first time that cannabidiol, and other cannabinoids, can be used as antioxidant drugs in the treatment (including prophylaxis) of oxidation associated diseases, and is particularly useful as a neuroprotectant. The therapeutic potential of nonpsychoactive cannabinoids is particularly promising, because of the absence of psychotoxicity, and the ability to administer higher doses than with psychotropic cannabinoids, such as THC.
Previous studies have also indicated that cannabidiol is not toxic, even when chronically administered to humans or given in large acute doses (700 mg/day).

EXAMPLE $
Effect of Cannabidiol on Lipoxygenase Enzymes This example describes in vitro and in vivo assays to examine the effect of cannabidiol (CBD) on three lipoxygenase (LO) enzymes: S-LO, 12-LO and 1S-LO.
S
In vitro Enzyme assay The ability of CBD to inhibit lipoxygenase was examined by measuring the time dependent change in absorption at 234 nM following addition of SU of each lipoxygenase (rabbit 1S-LO purchased from Biomol (PA), porcine 12-LO purchased from Cayman chemicals (MI)) to a solution containing 10 ~,M (final concentration) linoleic acid.
Enzyme studies were performed using a u.v. spectrophotometer and a 3 ml quartz cuvette containing 2.5 ml of a stirred solution of 12.5 ~M sodium linoleic acid (sodium salt) in solution A (2S mM Tris (pH 8.1), 1 mM EDTA 0.1 % methyl cellulose). The reaction was initiated by addition of O.S ml enzyme solution (10 U/ml enzyme in solution A) and recorded for 60 seconds. Lipoxygenase exhibits non-Michaelis-Menten kinetics, an initial "lag"
(priming) phase followed by a linear phase which is terminated by product inhibition. These complications were reduced by assessing enzyme activity (change in absorption) over the "steepest° 20 second period in a 60 second run time. Recordings examined the absorption at 234 nm minus the value at a reference wavelength of 280 nm. Linoleic acid was used as the substrate rather than arachidonic acid, because the products are less inhibitory to the enzyme, thereby providing a longer "linear phase" .
Cell purification and separation Human platelets and leukocytes were purified from huffy coat preparations (NIH
2S Blood Bank) using a standard Ficoll based centrifugation method used in blood banks. Prior to use, cells were washed three times to eliminate contaminating cell types. Cultured rat basophillic leukemia cells (RBL-2H3) were used as a source of S-lipoxygenase.

In vivo Determination of Lipoxygenase activity Cells were incubated with arachidonic acid and stimulated with the calcium ionophore A23187. Lipids were extracted and separated by reverse phase HPLC.
Product formation was assessed as the area of a peak that co-eluted with an authentic standard, had a greater absorbance at 236 nm than at either 210 or 280 nm, and the formation of which was inhibited by a lipoxygenase inhibitor.
Cell pellets were triturated in DMEM culture media, aliquoted and pre-incubated for 1S minutes with 20 teM arachidonic acid and varying concentrations of cannabidiol and/or 40 p.M nordihydroguaiaretic acid (a lipxygenase inhibitor). Platelets and leukocytes were also pre-incubated with 80 ~cM manoalide (Biomol) to prevent phospholipase A2 activation. Product formation was initiated by addition of 5 pM A23187 and incubation for 10 minutes at 37°C. At the end of the incubation, the reaction was stopped by addition of 15% 1M HCl and 10 ng/ml prostaglandin B2 (internal standard). Lipids were extracted with 1 volume of ethyl ether, which was dried under a stream of nitrogen. Samples were reconstituted in 50%
acetonitrile:50% H20 and separated by reverse phase HPLC using a gradient running from 63 %
acetonitrile: 37 Hz0:0.2% acetic acid to 90% acetonitrile (0.2% acetic acid) over 13 minutes.
Measurement of NMDAr toxicity The ability of 12-HETE (12-(s)-hydroxy-eicosatetraenoic acid, the product of the action of 12-lipoxygenase on arachidonic (eicosatetraenoic) acid) to protect cortical neurons from NMDAr toxicity was measured as described in Example 3. The 12-HETE (0.5 ~,g/ml) was added either during ischemia (co-incubated with the glutamate), during post-ischemia (co-incubated with the DMEM after washing the cells), or during both ischemia and post-ischemia.
Results Using semi-purified enzyme preparations, the effect of CBD on rabbit 15-LO and porcine 12-LO was compared. As shown in FIGS. 6A and B, CBD is a potent competitive inhibitor of 15-LO with an ECso of 598 nM. However, CBD had no effect on the 12-LO enzyme.
Using whole cell preparations, the effect of'CBD on 5- and 12-LO enzymes was investigated. As shown in FIG. 7A, CBD inhibited S-LO in cultured rat basophillic leukemia cells (ltBL-2H3) with an ECso of 1.92 ~.M. However, CBD had no effect on 12-LO, as monitored by the production of 12-HETE (the product of 12-LO), in either human leukocytes or platelets (FIGS.
7B and C). The leukocyte 12-LO is similar, while the platelet 12-LO is structurally and functionally different, from the porcine 12-LO used in the in vitro enzyme study.
The ability of 12-HETE to protect cortical neurons from NMDAr toxicity is shown in FIG. 8. To achieve best protection from NMDAr toxicity, 12-HETE was administered both during and post ischemia.
Therefore, CBD serves as a selective inhibitor of at least two lipoxygenase enzymes, 5-LO and 15-LO, but had no effect on 12-LO. Importantly, this is the first demonstration (FIG. 8) that the 12-LO product 12-HETE can play a significant role in protecting neurons from NMDAr mediated toxicity. Although the mechanism of this protection is unknown at the present time, 12-HETE is known to be an important neuromodulator, due to its ability to influence potassium channel activity.

WO 99/53917 PC'T/US99/08769 Methods of Treatment The present invention includes a treatment that inhibits oxidation associated diseases in a subject such as an animal, for example a rat or human. The method includes administering the antioxidant drugs of the present invention, or a combination of the antioxidant drug and one or more other pharmaceutical agents, to the subject in a pharmaceutically compatible carrier and in an effective amount to inhibit the development or progression of oxidation associated diseases.
Although the treatment can be used prophylactically in any patient in a demographic group at significant risk for such diseases, subjects can also be selected using more specific criteria, such as a definitive diagnosis of the condition. The administration of any exogenous antioxidant cannabinoid would inhibit the progression of the oxidation associated disease as compared to a subject to whom the cannabinoid was not administered. The antioxidant effect, however, increases with the dose of the cannabinoid.
The vehicle in which the drug is delivered can include pharmaceutically acceptable compositions of the drugs of the present invention using methods well known to those with skill in the ari. Any of the common carriers, such as sterile saline or glucose solution, can be utilized with the drugs provided by the invention. Routes of administration include but are not limited to oral, intracranial ventricular (icv), intrathecal (it), intravenous (iv), parenteral, rectal, topical ophthalmic, subconjunctival, nasal, aural, sub-lingual (under the tongue) and transdermal. The antioxidant drugs of the invention may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, lipid carriers such as cyclodextrins, proteins such as serum albumin, hydrophilic agents such as methyl cellulose, detergents, buffers, preservatives and the like. Given the Iow solubility of many cannabinoids, they may be suspended in sesame oil.
Given the excellent absorption of the compounds of the present invention via an inhaled route, the compounds may also be administered as inhalants, for example in pharmaceutical aerosols utilizing solutions, suspensions, emulsions, powders and semisolid preparations of the type more fully described in Remington: The Science and Practice of Pharnracy (19'"
Edition, 1995) in chapter 95. A particular inhalant form is a metered dose inhalant containing the active ingredient, in a suspension or a dispersing agent (such as sorbitan trioleate, oleyl alcohol, oleic acid, or lecithin, and a propellant such as 12/11 or 12/114).
Embodiments of the invention comprising pharmaceutical compositions can be prepared with conventional pharmaceutically acceptable carriers, adjuvants and counterions as would be known to those of skill in the art. The compositions are preferably in the form of a unit dose in solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, injectable and infusible solutions, for example a unit dose vial, or a metered dose inhaler. Effective oral human dosage ranges for cannabidiol are contemplated to vary from about 1-40 mg/kg, for example 5-20 mg/kg, and in particular a dose of about 20 mg/kg of body weight.
If the antioxidant drugs are to be used in the prevention of cataracts, they may be administered in the form of eye drops formulated in a pharmaceutically inert, biologically acceptable carrier, such as isotonic saline or an ointment. Conventional preservatives, such as benzalkonium chloride, can also be added to the formulation. In ophthalmic ointments, the active ingredient is admixed with a suitable base, such as white petrolatum and mineral oil, along with antimicrobial preservatives. Specific methods of compounding these dosage forms, as well as appropriate pharmaceutical carriers, are known in the art. Remington: The Science and Practice of Pharmacy , 19th Ed., Mack Publishing Co. (1995), particularly Part 7.
The compounds of the present invention are ideally administered as soon as a diagnosis is made of an ischemic event, or other oxidative insult. For example, once a myocardial infarction has been confirmed by electrocardiograph, or an elevation in enzymes characteristic of cardiac injury (e.g. CKMB), a therapeutically effective amount of the cannabinoid drug is administered. A dose can also be given following symptoms characteristic of a stroke (motor or sensory abnormalities), or radiographic confirmation of a cerebral infarct in a distribution characteristic of a neurovascular thromboembolic event. The dose can be given by frequent bolus administration, or as a continuous 1V dose. In the case of cannabidiol, for example, the drug could be given in a dose of 5 mg/kg active ingredient as a continuous intravenous infusion; or hourly intramuscular injections of that dose.

The following table lists examples of some dibenzopyran cannabinoids that may be useful as antioxidants in the method of the present invention.
R: R.

Compound R,9 R ~ R" Rz, RZ~ R 4 R2s R2s H 7-OH-0'-THC CH20H H H H H H H CSH"

H 6a-OH-~'-THC CH3 a-OH
_6 H 6~i-OH-D'-THC CH3 (3-OH
8 1"-OH-D'-THC CH3 OH

H 2"-OH-0'-THC CH3 OH

_9 _10 3 "-OH-D'-THC CH3 OH

_ll 4"-OH-D'-THC CH3 OH

H 6a,7-diOH-A'-THC CHZOH a-OH
_12 H 6v,7-diOH-D'-THE CHZOH ~i-OH
_13 _14 1",7-diOH-O'-THC CHZOH OH

H 2",7-diOH-0'-THC CHZOH OH
_15 H 3",7-diOH-A'-THC CH20H OH
_16 H 4",7-diOH-0'-THC CHZOH OH
_I7 _18 1",6(3-diOH-D'-THCCH3 (3-OH OH

_19 1",3"-diOH-A'-THCCH3 OH OH

_20 1 ",6C~,7-triOH-A'-THCCHZOH a-OH OH

H 0'-THG6-one CH3 =0 _21 _22 EpoxyhexahydrocannabinolCH3 (EHHC)' _23 7-oxo-0'-THC CHO

H 0'-THC-7-oic acidCOOH

H 0'-THC-3"-oic CH3 C2H4COOH
25 acid -H 1 "-OH-A'-THC-7"-oicCOOH OH
_26 acid H 2"-OH-0'-THC-7"-oicCOOH OH
_27 acid H 3"-OH-D'-THC-7"-oicCOOH OH
_28 acid H 4"-OH-0'-THC-7"-oicCOOH OH
_29 acid H 3",4',5'-trisnor-2'-OH-D'-COOH C2H,OH
_30 THC-7-oic acid H 7-OH-0'-THC-2"-oicCHZOH CHzCOOH
31 acid H 6(i-OH-0'-THC-2'-oicCH3 (i-OH CHZCOOH
32 acid -H 7-OH-A'-THC-3'-oicCHZOH CZH4COOH
_33 acid H 6(3-OH-D'-THC-3"-oicCH3 (i-OH CZH4COOH
34 acid H 6a-OH-0'-THC-4'-oicCH3 a-OH C3H6COOH
35 acid -H 2",3"-dehydro-60-OH-0'-CH3 a-OH C,H,COOH
_36 THC-4"-.oic acid H 0'-THC-1",7-dioicCOOH COOH
37 acid H 0'-THC-2",7-dioicCOOH CH2COOH
38 acid -H D'-THC-3",7-dioicCOOH CZH,COOH
_ acid H 0'-THC-4",7-dioicCOOH C3HsCOOH
_40 acid H 1",2"-dehydro-0'-THC-3',7-COOH CZHzCOOH

dioic acid H 0'-THC-glucuronicCH3 gluct 42 acid -H t1'-THC-7-oic COO gluct _43 acid elucuronide Epoxy group in C-1 and C-2 positions t Glucuronide Note: R-group substituents are H if not indicated otherwise Chemical structures of some of the dibenzopyran cannabinoids are shown below.

Examples of Structural Analogs of Cannabidiol The following table lists examples of some cannabinoids which are structural analogs of cannabidiol and that may be useful as antioxidants in the method of the present invention. A particularly useful example is compound CBD, cannabidiol.

R22 Rz4 Compound R,9 Rio Rz, Rzz Rz; Rza Rzs Rzb 44 CBD CH3 H H H H H H CsH"

45 7-OH-CBD CHzOH

46 6a- CH3 a-OH

47 6(3- CH3 (3-OH

48 1 "- CH3 OH

49 2"- CH3 OH

SO 3"- CH3 OH

51 4"- CH3 OH

52 5 "- CH3 C,H8CH20H

53 6,7-diOH-CBDCHZOH OH

54 3",7-diOH-CHZOH OH

CBD

55 4",7-diOH-CHzOH OH

CBD

56 CBD-7-oicCOOH
acid 57 CBD-3"-oicCH3 CZH4COOH

acid Note: R-group substituents are H if not indicated otherwise.

Compound R,9 Rzo RZ, R22 Rz3 Rza Rzs Rz6 58 CBN CH3 H H H H H H CsH"

60 1 "-OH-CBNCH3 OH

612"-OH-CBN CH; OH

62 3"-OH-CBNCH3 OH

63 4"-OH-CBNCH3 OH

64 5"-OH-CBNCH3 C,HgCH20H

65 2"-7-diOH-CHZOH OH

CBN

66 CBN-7-oicCOOH
acid "-oic acid 68 CBN-3"-oicCH3 C2H4COOH

acid Note: R-group substituents are H if not indicated otherwise.
The invention being thus described, variation in the materials and methods for practicing the invention will be apparent to one of ordinary skill in the art.
Such variations are to be considered within the scope of the invention, which is set forth in the claims below.
Classifications
International Classification G01N33/15, A61P9/00, A61K31/05, A61K31/35, C07D311/80, A61P25/16, A61K31/12, A61K31/352, A61P25/00, A61K31/09, A61P43/00, A61P39/06, A61P25/28
Cooperative Classification A61K31/35
European Classification A61K31/35
Legal Events
Date Code Event Description
Apr 21, 2005 FZDE Dead

From <http://www.google.com/patents/CA2329626A1?cl=en>

37. Cannabinoids As Antioxidants And Neuroprotectants
Cannabinoids have been found to have antioxidant properties, unrelated to NMDA receptor antagonism. This new found property makes cannabinoids useful in the treatment and prophylaxis of wide variety of oxidation associated diseases, such as ischemic, age-related, inflammatory and autoimmune diseases. The cannabinoids are found to have particular application as neuroprotectants, for example in limiting neurological damage following ischemic insults, such as stroke and trauma, or in the treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and HIV dementia. Nonpsychoactive cannabinoids, such as cannabidoil, are particularly advantageous to use because they avoid toxicity that is encountered with psychoactive cannabinoids at high doses useful in the method of the present invention. A particular disclosed class of cannabinoids useful as neuroprotective antioxidants is formula (I) wherein the R group is independently selected from the group consisting of H, CH3, and COCH3. ##STR1##
Claims

We claim:

1. A method of treating diseases caused by oxidative stress, comprising administering a therapeutically effective amount of a cannabinoid that has substantially no binding to the NMDA receptor to a subject who has a disease caused by oxidative stress.

2. The method of claim 1, wherein the cannabinoid is nonpsychoactive.

3. The method of claim 2, wherein the cannabinoid has a volume of distribution of 10 L/kg or more.

4. The method of claim 1, wherein the cannabinoid is not an antagonist at the NMDA receptor.

5. The method of claim 1, wherein the cannabinoid is: ##STR22##

where R is H, substituted or unsubstituted alkyl, carboxyl, alkoxy, aryl, aryloxy, arylalkyl, halo or amino.

6. The method of claim 5, wherein R is H, substituted or unsubstituted alkyl, carboxyl or alkoxy.

7. The method of claim 2, wherein the cannabinoid is: ##STR23##

where

A is cyclohexyl, substituted or unsubstituted aryl, or ##STR24##

but not a pinene;

R1 is H, substituted or unsubstituted alkyl, or substituted or unsubstituted carboxyl;

R2 is H, lower substituted or unsubstituted alkyl, or alkoxy;

R3 is of H, lower substituted or unsubstituted alkyl, or substituted or unsubstituted carboxyl;

R4 is H, hydroxyl, or lower substituted or unsubstituted alkyl; and

R5 is H, hydroxyl, or lower substituted or unsubstituted alkyl.

8. The method of claim 7, wherein

R1 is lower alkyl, COOH or COCH3 ;

R2 is unsubstituted C1 -C5 alkyl, hydroxyl, methoxy or ethoxy;

R3 is H, unsubstituted C1 -C3 alkyl, or COCH3 ;

R4 is hydroxyl, pentyl, heptyl, or diemthylheptyl; and

R5 is hydroxyl or methyl.

9. The method of claim 1, wherein the cannabinoid is: ##STR25##

where R1, R2 and R3 are independently H, CH3, or COCH3.

10. The method of claim 9, wherein the cannabinoid is: ##STR26##

where:

a) R1 =R2 =R3 =H;

b) R1 =R3 =H, R2 =CH3 ;

c) R1 =R2 =CH3, R3 =H;

d) R1 =R2 =COCH3, R3 =H; or

e) R1 =H, R2 =R3 =COCH3.

11. The method of claim 2, wherein the cannabinoid is: ##STR27##

where R19 is H, lower alkyl, lower alcohol, or carboxyl; R20 is H or OH; and R21 -R25 are independently H or OH.

12. The method of claim 11, wherein R19 is H, CH3, CH2 OH, or COOH, and R20 -R24 are independently H or OH.

13. The method of claim 2, wherein the cannabinoid is: ##STR28##

where R19 and R20 are H, and R26 is alkyl.

14. The method of claim 10, wherein the cannabinoid is cannabidiol.

15. A method of treating an ischemic or neurodegenerative disease in the central nervous system of a subject, comprising administering to the subject a therapeutically effective amount of a cannabinoid, where the cannabinoid is ##STR29##

where R is H, substituted or unsubstituted alkyl, carboxyl, alkoxy, aryl, aryloxy, arylalkyl, halo or amino.

16. The method of claim 15, wherein the cannabinoid is not a psychoactive cannabinoid.

17. The method of claim 15 where the ischemic or neurodegenerative disease is an ischemic infarct, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus dementia, Down's syndrome, or heart disease.

18. A method of treating a disease with a cannabinoid that has substantially no binding to the NMDA receptor, comprising determining whether the disease is caused by oxidative stress, and if the disease is caused by oxidative stress, administering the cannabinoid in a therapeutically effective antioxidant amount.

19. The method of claim 18, wherein the cannabinoid has a volume of distribution of at least 1.5 L/kg and substantially no activity at the cannabinoid receptor.

20. The method of claim 19, wherein the cannabinoid has a volume of distribution of at least 10 L/kg.

21. The method of claim 1, wherein the cannabinoid selectively inhibits an enzyme activity of 5- and 15-lipoxygenase more than an enzyme activity of 12-lipoxygenase.

22. A method of treating a neurodegenerative or ischemic disease in the central nervous system of a subject, comprising administering to the subject a therapeutically effective amount of a compound selected from any of the compounds of claims 9 through 13.

23. The method of claim 22 where the compound is cannabidiol.

24. The method of claim 22, wherein the ischemic or neurodegenerative disease is an ischemic infarct, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus dementia, Down's syndrome, or heart disease.

25. The method of claim 24 wherein the disease is an ischemic infarct.

26. The method of claim 1, wherein the cannabinoid is not an antagonist at the AMPA receptor.

Description

FIELD OF THE INVENTION

The present invention concerns pharmaceutical compounds and compositions that are useful as tissue protectants, such as neuroprotectants and cardioprotectants. The compounds and compositions may be used, for example, in the treatment of acute ischemic neurological insults or chronic neurodegenerative diseases.

BACKGROUND OF THE INVENTION

Permanent injury to the central nervous system (CNS) occurs in a variety of medical conditions, and has been the subject of intense scientific scrutiny in recent years. It is known that the brain has high metabolic requirements, and that it can suffer permanent neurologic damage if deprived of sufficient oxygen (hypoxia) for even a few minutes. In the absence of oxygen (anoxia), mitochondrial production of ATP cannot meet the metabolic requirements of the brain, and tissue damage occurs. This process is exacerbated by neuronal release of the neurotransmitter glutamate, which stimulates NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) and kainate receptors. Activation of these receptors initiates calcium influx into the neurons, and production of reactive oxygen species, which are potent toxins that damage important cellular structures such as membranes, DNA and enzymes.

The brain has many redundant blood supplies, which means that its tissue is seldom completely deprived of oxygen, even during acute ischemic events caused by thromboembolic events or trauma. A combination of the injury of hypoxia with the added insult of glutamate toxicity is therefore believed to be ultimately responsible for cellular death. Hence if the additive insult of glutamate toxicity can be alleviated, neurological damage could also be lessened. Anti-oxidants and anti-inflammatory agents have been proposed to reduce damage, but they often have poor access to structures such as the brain (which are protected by the blood brain barrier).

Given the importance of the NMDA, AMPA and kainate receptors in the mechanism of injury, research efforts have focused on using antagonists to these receptors to interfere with the receptor mediated calcium influx that ultimately leads to cellular death and tissue necrosis. In vitro studies using cultured neurons have demonstrated that glutamate receptor antagonists reduce neurotoxicity, but NMDA and AMPA/kainate receptor antagonists have different effects. Antagonists to NMDAr prevent neurotoxicity if present during the glutamate exposure period, but are less effective if added after glutamate is removed. In contrast, AMPA/kainate receptor antagonists are not as effective as NMDA antagonists during the glutamate exposure period, but are more effective following glutamate exposure.

Some of the research on these antagonists has focused on cannabinoids, a subset of which have been found to be NMDA receptor antagonists. U.S. Pat. No. 5,538,993 (3S,4S-delta-6-tetrahydrocannabinol-7-oic acids), U.S. Pat. No. 5,521,215 (sterospecific ( ) THC enantiomers), and U.S. Pat. No. 5,284,867 (dimethylheptyl benzopyrans) have reported that these cannabinoids are effective NMDA receptor blockers. U.S. Pat. No. 5,434,295 discloses that the 1,1 dimethylheptyl (DMH) homolog of [3R,4R]-7-hydroxy-Δ6 THC (known as HU-210) is a superpotent cannabinoid receptor agonist with cannabinomimetic activity two orders of magnitude greater than the natural Δ9 THC. The HU-210 dimethylheptyl cannabinoid, has severe side effects, including fatigue, thirst, headache, and hypotension. J. Pharmacol. Sci. 60:1433-1457 (1971). Subjects who received this synthetic cannabinoid with a dimethylheptyl group experienced marked psychomotor retardation, and were unwilling or incapable of assuming an erect position.

In contrast to HU-210, the (-)(3R,4R) THC-DMH enantiomer (known as HU-211) displays low affinity to the cannabinoid receptors, but retains NMDA receptor antagonist neuroprotective activity. ##STR2##

THC (tetrahydrocannabinol) is another of the cannabinoids that has been shown to be neuroprotective in cell cultures, but this protection was believed to be mediated by interaction at the cannabinoid receptor, and so would be accompanied by undesired psychotropic side effects. ##STR3##

Although it has been unclear whether cannabimimetic activity plays a role in neuroprotection against glutamate induced neurological injury, the teaching in this field has clearly been that a cannabinoid must at least be an antagonist at the NMDA receptor to have neuroprotective effect. Hence cannabidiol (2-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenedi ol or CBD), a cannabinoid devoid of psychoactive effect (Pharm. Rev. 38:21-43, 1986), has not been considered useful as a neuroprotectant. Cannabidiol has been studied as an antiepileptic (Carlini et al., J. Clin. Pharmacol. 21:417S-427S, 1981; Karler et al., J. Clin. Pharmacol. 21:437S-448S, 1981, Consroe et al., J. Clin Phannacol. 21:428S-436S, 1981), and has been found to lower intraocular pressure (Colasanti et al, Exp. Eye Res. 39:251-259, 1984 and Gen. Pharmac. 15:479-484, 1984). ##STR4##

No signs of toxicity or serious side effects have been observed following chronic administration of cannabidiol to healthy volunteers (Cunha et al., Pharmacology 21:175-185, 1980), even in large acute doses of 700 mg/day (Consroe et al., Pharmacol. Biochem. Behav. 40:701-708, 1991) but cannabidiol is inactive at the NMDA receptor. Hence in spite of its potential use in treating glaucoma and seizures, cannabidiol has not been considered a neuroprotective agent that could be used to prevent glutamate induced damage in the central nervous system.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a new class of antioxidant drugs, that have particular application as neuroprotectants, although they are generally useful in the treatment of many oxidation associated diseases.

Yet another object of the invention is to provide a subset of such drugs that can be substantially free of psychoactive or psychotoxic effects, are substantially non-toxic even at very high doses, and have good tissue penetration, for example crossing the blood brain barrier.

It has surprisingly been found that cannabidiol and other cannabinoids can function as neuroprotectants, even though they lack NMDA receptor antagonist activity. This discovery was made possible because of the inventor's recognition of a previously unanticipated antioxidant property of the cannabinoids in general (and cannabidiol in particular) that functions completely independently of antagonism at the NMDA, AMPA and kainate receptors. Hence the present invention includes methods of preventing or treating diseases caused by oxidative stress, such as neuronal hypoxia, by administering a prophylactic or therapeutically effective amount of a cannabinoid to a subject who has a disease caused by oxidative stress.

The cannabinoid may be a cannabinoid other than THC, HU-210, or other potent cannabinoid receptor agonists. The cannabinoid may also be other than HU-211 or any other NMDA receptor antagonist that has previously been reported. A potent cannabinoid receptor agonist is one that has an EC50 at the cannabinoid receptor of 50 nM or less, but in more particular embodiments 190 nM or 250 nM or less. In disclosed embodiments the cannabinoid is not psychoactive, and is not psychotoxic even at high doses. In some particularly disclosed embodiments, the cannabinoid is selected from the group: ##STR5##

where A is aryl, and particularly ##STR6##

but not a pinene such as: ##STR7##

and the R1 -R5 groups are each independently selected from the groups of hydrogen, lower substituted or unsubstituted alkyl, substituted or unsubstituted carboxyl, substituted or unsubstituted alkoxy, substituted or unsubstituted alcohol, and substituted or unsubstituted ethers, and R6 -R7 are H or methyl. In particular embodiments, there are no nitrogens in the rings, and/or no amino substitutions on the rings.

In other embodiments, the cannabinoid is one of the following: ##STR8##

where there can be 0 to 3 double bonds on the A ring, as indicated by the optional double bonds indicated by dashed lines on the A ring. The C ring is aromatic, and the B ring can be a pyran. Particular embodiments are dibenzo pyrans and cyclohexenyl benzenediols. Particular embodiments of the cannabinoids of the present invention may also be highly lipid soluble, and in particular embodiments can be dissolved in an aqueous solution only sparingly (for example 10 mg/ml or less). The octanol/water partition ratio at neutral pH in useful embodiments is 5000 or greater, for example 6000 or greater. This high lipid solubility enhances penetration of the drug into the CNS, as reflected by its volume of distribution (Vd) of 1.5 L/kg or more, for example 3.5 L/kg, 7 L/kg, or ideally 10 L/kg or more, for example at least 20 L/kg. Particular embodiments may also be highly water soluble derivatives that are able to penetrate the CNS, for example carboxyl derivatives.

R7-18 are independently selected from the group of H, substituted or unsubstituted alkyl, especially lower alkyl, for example unsubstituted C1 -C3 alkyl, hydroxyl, alkoxy, especially lower alkoxy such as methoxy or ethoxy, substituted or unsubstituted alcohol, and unsubstituted or substituted carboxyl, for example COOH or COCH3. In other embodiments R7-18 can also be substituted or unsubstituted amino, and halogen.

The cannabinoid has substantially no binding to the NMDAr (for example an IC50 greater than or equal to 5 μM or 10 μM), has substantially no psychoactive activity mediated by the cannabinoid receptor (for example an IC50 at the cannabinoid receptor of greater than or equal to 300 nM, for example greater than 1 μM and a Ki greater than 250 nM, especially 500-1000 nM, for example greater than 1000 nM), and antioxidant activity, as demonstratable by the Fenton reaction or cyclic voltametry.

In other particular embodiments, the cannabinoids are one of the following: ##STR9##

where R19 is substituted or unsubstituted alkyl, such as lower alkyl (for example methyl), lower alcohol (such as methyl alcohol) or carboxyl (such as carboxylic acid) and oxygen (as in =O); R20 is hydrogen or hydroxy; R21 is hydrogen, hydroxy, or methoxy; R22 is hydrogen or hydroxy; R23 is hydrogen or hydroxy; R24 is hydrogen or hydroxy; R25 is hydrogen or hydroxy; and R26 is substituted or unsubstituted alkyl (for example n-methyl alkyl), substituted or unsubstituted alcohol, or substituted or unsubstituted carboxy.

In yet other embodiments of the invention, the cannabinoids are ##STR10##

wherein numbering conventions for each of the ring positions are shown, and R27, R28 and R29 are independently selected from the group consisting of H, unsubstituted lower alkyl such as CH3, and carboxyl such as COCH3. Particular examples of nonpsychoactive cannabinoids that fall within this definition are cannabidiol and ##STR11##

and other structural analogs of cannabidiol.

In more particular embodiments, the cannabinoid is used to prevent or treat an ischemic or neurodegenerative disease in the central nervous system of a subject, by administering to the subject a therapeutically effective amount of a cannabinoid to protect against oxidative injury to the central nervous system. The cannabinoid may be any of the compounds set forth above, or more specifically ##STR12##

wherein R27, R28 and R29 are independently selected from the group consisting of H, lower alkyl such as CH3, and carboxyl such as COCH3, and particularly wherein

a) R27 =R28 =R29 =H

b) R27 =R29 =H; R28 =CH3

c) R27 =R28 =CH3 ; R29 =H

d) R27 =R28 =COCH3 ; R29 =H

e) R27 =H; R28 =R29 =COCH3

When R27 =R28 =R29 =H, then the compound is cannabidiol. When R27 =R29 =H and R28 =CH3, the compound is CBD monomethyl ether. When R27 =R28 =CH3 and R29 =H, the compound is CBD dimethyl ether. When R27 =R28 =COCH3 and R29 =H, the compound is CBD diacetate. When R27 =H and R28 =R29 =COCH3, the compound is CBD monoacetate. The ischemic or neurodegenerative disease may be, for example, an ischemic infarct, Alzheimer's disease, Parkinson's disease, Down's syndrome, human immunodeficiency virus (HIV) dementia, myocardial infarction, or treatment and prevention of intraoperative or perioperative hypoxic insults that can leave persistent neurological deficits following open heart surgery requiring heart/lung bypass machines, such as coronary artery bypass grafts (CABG).

The invention also includes an assay for selecting a cannabinoid to use in treating a neurological disease by determining whether the cannabinoid is an antioxidant. Once it has been determined that the cannabinoid is an antioxidant, an antioxidant effective amount of the cannabinoid is administered to treat the neurological disease, such as a vascular ischemic event in the central nervous system, for example the type caused by a neurovascular thromboembolism. Similarly, the method of the present invention includes determining whether a disease is caused by oxidative stress, and if the disease is caused by oxidative stress, administering the cannabinoid in a therapeutically effective antioxidant amount.

The invention also includes identifying and administering antioxidant and neuroprotective compounds (such as cannabidiol) which selectively inhibit the enzyme activity of both 5- and 15-lipoxygenase more than the enzyme activity of 12-lipoxygenase. In addition, such compounds posses low NMDA antagonist activity and low cannabinoid receptor activity. Assays for selecting compounds with the desired effect on lipoxygenase enzymes, and methods for using identified compounds to treat neurological or ischemic diseases are also provided. Such diseases may include a vascular ischemic event in the central nervous system, for example a thromboembolism in the brain, or a vascular ischemic event in the myocardium. Useful administration of the compounds involves administration both during and after an ischemic injury.

These and other objects of the invention will be understood more clearly by reference to the following detailed description and drawings.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A is a graph showing NMDA induced cellular damage in a neuron (as measured by LDH release) in cells that were exposed to glutamate for 10 minutes, which demonstrates that increasing concentrations of cannabidiol in the cell culture protects against cellular damage.

FIG. 1B is a graph similar to FIG. 1A, but showing that AMPA/kainate receptor mediated damage (induced by glutamate and the AMPA/kainate receptor potentiating agents cyclothiazide or concanavalin A) is also reduced in a concentration dependent manner by the presence of cannabidiol in the culture medium.

FIG. 2A is a bar graph showing cellular damage (as measured by LDH release) in the presence of glutamate alone (100 μM Glu), and in the presence of glutamate and 5 μM cannabidiol (CBD) or 5 μM THC, and demonstrates that CBD and THC were similarly protective.

FIG. 2B is a bar graph similar to FIG. 2A, but showing the cellular damage assessed in the presence of the cannabinoid receptor antagonist SR 141716A (SR), which was not found to alter the neuroprotective effect of CBD (5 μM) or THC (5 μM), indicating the effect is not a typical cannabinoid effect mediated by the cannabinoid receptor.

FIG. 3 is a graph showing the reduction oxidation potentials determined by cyclic voltametry for some natural and synthetic cannabinoids, the antioxidant BHT, and the non-cannabinoid anandamide (arachidonyl ethanolamide) which is a ligand for the cannabinoid receptor. The voltage at which initial peaks occur is an indication of antioxidant activity.

FIG. 4 is a graph that demonstrates the antioxidant properties of BHT, CBD and THC, by plotting the fluorescence of a fluorescent dye against concentrations of these substances, where declining fluorescence is an indication of greater antioxidant activity.

FIG. 5A is a graph illustrating decreased t-butyl peroxide induced toxicity (as measured by LDH release) in the presence of increasing concentrations of cannabidiol, demonstrating that cannabidiol is an effective antioxidant in living cells.

FIG. 5B is a bar graph comparing the antioxidant activity of several antioxidants against glutamate induced toxicity in neurons, showing that CBD has superior antioxidant activity.

FIG. 6A is a graph showing the effect of CBD (as measured by the change in absorbance at 234 nm) on the enzymatic activity of two lipoxygenase enzymes, rabbit 15-LO and porcine 12-LO, which demonstrates that CBD inhibits 15-LO, but not 12-LO enzyme.

FIG. 6B is a graph demonstrating that inhibitory effect of CBD on 15-LO is competitive.

FIG. 7A is a graph similar to FIG. 6A, but was performed in whole cells rather than purified enzyme preparations, and shows the effect of CBD (as measured by the change in absorbance at 236 nm) on the enzymatic activity of 5-LO from cultured rat basophillic leukemia cells (RBL-2H3), which demonstrates that CBD inhibits 5-LO.

FIG. 7B is a graph showing the effect of CBD (as measured by the change in absorbance at 236 nm) on the formation of 12-HETE (the product of 12-LO) by human leukocytes (12-LO type 1).

FIG. 7C is a graph similar to FIG. 7B, showing the effect of CBD (as measured by the change in absorbance at 236 nm) on the formation of 12-HETE by human platelets (12-LO type 2).

FIG. 8 is a bar graph demonstrating that 12-HETE can protect cortical neurons from NMDAr toxicity most effectively when administered during and post ischemia.

DETAILED DESCRIPTION OF SOME SPECIFIC EMBODIMENTS

This invention provides antioxidant compounds and compositions, such as pharmaceutical compositions, that include cannabinoids that act as free radical scavengers for use in prophylaxis and treatment of disease. The invention also includes methods for using the antioxidants in prevention and treatment of pathological conditions such as ischemia (tissue hypoxia), and in subjects who have been exposed to oxidant inducing agents such as cancer chemotherapy, toxins, radiation, or other sources of oxidative stress. The compositions and methods described herein are also used for preventing oxidative damage in transplanted organs, for inhibiting reoxygenation injury following reperfusion of ischemic tissues (for example in heart disease), and for any other condition that is mediated by oxidative or free radical mechanisms of injury. In particular embodiments of the invention, the compounds and compositions are used in the treatment of ischemic cardiovascular and neurovascular conditions, and neurodegenerative diseases. However the present invention can also be used as an antioxidant treatment in non-neurological diseases.

Molecular oxygen is essential for aerobic organisms, where it participates in many biochemical reactions, including its role as the terminal electron acceptor in oxidative phosphorylation. However excessive concentrations of various forms of reactive oxygen species and other free radicals can have serious adverse biological consequences, including the peroxidation of membrane lipids, hydroxylation of nucleic acid bases, and the oxidation of sulfhydryl groups and other protein moieties. Biological antioxidants include tocopherols and tocotrieneols, carotenoids, quinones, bilirubin, ascorbic acid, uric acid, and metal binding proteins. However these endogenous antioxidant systems are often overwhelmed by pathological processes that allow permanent oxidative damage to occur to tissue.

Free radicals are atoms, ions or molecules that contain an unpaired electron, are usually unstable, and exhibit short half-lives. Reactive oxygen species (ROS) is a collective term, designating the oxygen radicals (e.g. .O2- superoxide radical), which by sequential univalent reduction produces hydrogen peroxide (H2 O2) and hydroxyl radical (.OH). The hydroxyl radical sets off chain reactions and can interact with nucleic acids. Other ROS include nitric oxide (NO.) and peroxy nitrite (NOO.), and other peroxyl (RO2.) and alkoxyl (RO.) radicals. Increased production of these poisonous metabolites in certain pathological conditions is believed to cause cellular damage through the action of the highly reactive molecules on proteins, lipids and DNA. In particular, ROS are believed to accumulate when tissues are subjected to ischemia, particularly when followed by reperfusion.

The pharmaceutical compositions of the present invention have potent antioxidant and/or free radical scavenging properties, that prevent or reduce oxidative damage in biological systems, such as occurs in ischemic/reperfusion injury, or in chronic neurodegenerative diseases such as Alzheimer's disease, HIV dementia, and many other oxidation associated diseases.

DEFINITIONS

"Oxidative associated diseases" refers to pathological conditions that result at least in part from the production of or exposure to free radicals, particularly oxyradicals, or reactive oxygen species. It is evident to those of skill in the art that most pathological conditions are multifactorial, and that assigning or identifying the predominant causal factors for any particular condition is frequently difficult. For these reasons, the term "free

radical associated disease" encompasses pathological states that are recognized as conditions in which free radicals or ROS contribute to the pathology of the disease, or wherein administration of a free radical inhibitor (e.g. desferroxamine), scavenger (e.g. tocopherol, glutathione) or catalyst (e.g. superoxide dismutase, catalase) is shown to produce detectable benefit by decreasing symptoms, increasing survival, or providing other detectable clinical benefits in treating or preventing the pathological state.

Oxidative associated diseases include, without limitation, free radical associated diseases, such as ischemia, ischemic reperfusion injury, inflammatory diseases, systemic lupus erythematosis, myocardial ischemia or infarction, cerebrovascular accidents (such as a thromboembolic or hemorrhagic stroke) that can lead to ischemia or an infarct in the brain, operative ischemia, traumatic hemorrhage (for example a hypovolemic stroke that can lead to CNS hypoxia or anoxia), spinal cord trauma, Down's syndrome, Crohn's disease, autoimmune diseases (e.g. rheumatoid arthritis or diabetes), cataract formation, uveitis, emphysema, gastric ulcers, oxygen toxicity, neoplasia, undesired cellular apoptosis, radiation sickness, and others. The present invention is believed to be particularly beneficial in the treatment of oxidative associated diseases of the CNS, because of the ability of the cannabinoids to cross the blood brain barrier and exert their antioxidant effects in the brain. In particular embodiments, the pharmaceutical composition of the present invention is used for preventing, arresting, or treating neurological damage in Parkinson's disease, Alzheimer's disease and HIV dementia; autoimmune neurodegeneration of the type that can occur in encephalitis, and hypoxic or anoxic neuronal damage that can result from apnea, respiratory arrest or cardiac arrest, and anoxia caused by drowning, brain surgery or trauma (such as concussion or spinal cord shock).

As used herein, an "antioxidant" is a substance that, when present in a mixture containing an oxidizable substrate biological molecule, significantly delays or prevents oxidation of the substrate biological molecule. Antioxidants can act by scavenging biologically important reactive free radicals or other reactive oxygen species (.O2-, H2 O2, .OH, HOCl, ferryl, peroxyl, peroxynitrite, and alkoxyl), or by preventing their formation, or by catalytically converting the free radical or other reactive oxygen species to a less reactive species. Relative antioxidant activity can be measured by cyclic voltametry studies of the type disclosed in Example 5 (and FIG. 3), where the voltage (x-axis) is an index of relative antioxidant activity. The voltage at which the first peak occurs is an indication of the voltage at which an electron is donated, which in turn is an index of antioxidant activity.

"Therapeutically effective antioxidant doses" can be determined by various methods, including generating an empirical dose-response curve, predicting potency and efficacy of a congener by using quantitative structure activity relationships (QSAR) methods or molecular modeling, and other methods used in the pharmaceutical sciences. Since oxidative damage is generally cumulative, there is no minimum threshold level (or dose) with respect to efficacy. However, minimum doses for producing a detectable therapeutic or prophylactic effect for particular disease states can be established.

As used herein, a "cannabinoid" is a chemical compound (such as cannabinol, THC or cannabidiol) that is found in the plant species Cannabis saliva (marijuana), and metabolites and synthetic analogues thereof that may or may not have psychoactive properties. Cannabinoids therefore include (without limitation) compounds (such as THC) that have high affinity for the cannabinoid receptor (for example Ki <250 nM), and compounds that do not have significant affinity for the cannabinoid receptor (such as cannabidiol, CBD). Cannabinoids also include compounds that have a characteristic dibenzopyran ring structure (of the type seen in THC) and cannabinoids which do not possess a pyran ring (such as cannabidiol). Hence a partial list of cannabinoids includes THC, CBD, dimethyl heptylpentyl cannabidiol (DMHP-CBD), 6,12-dihydro-6-hydroxy-cannabidiol (described in U.S. Pat. No. 5,227,537, incorporated by reference); (3S,4R)-7-hydroxy-Δ6 -tetrahydrocannabinol homologs and derivatives described in U.S. Pat. No. 4,876,276, incorporated by reference; ( )-4-[4-DMH-2,6-diacetoxy-phenyl]-2-carboxy-6,6-dimethylbicyclo[3.1. 1]hept-2-en, and other 4-phenylpinene derivatives disclosed in U.S. Pat. No. 5,434,295, which is incorporated by reference; and cannabidiol (-)(CBD) analogs such as (-)CBD-monomethylether, (-)CBD dimethyl ether; (-)CBD diacetate; (-)3'-acetyl-CBD monoacetate; and . -.AF11, all of which are disclosed in Consroe et al., J. Clin. Phannacol. 21:428S-436S, 1981, which is also incorporated by reference. Many other cannabinoids are similarly disclosed in Agurell et al., Pharmacol. Rev. 38:31-43, 1986, which is also incorporated by reference.

As referred to herein, the term "psychoactivity" means "cannabinoid receptor mediated psychoactivity." Such effects include, euphoria, lightheadedness, reduced motor coordination, and memory impairment. Psychoactivity is not meant to include non-cannabinoid receptor mediated effects such as the anxiolytic effect of CBD.

The "lipoxygenase enzyme activity" refers to the relative level of lipoxygenase enzyme activity for a particular lipoxgenase, such as 5-, 15- or 12-lipoxygenase, as measured in Example 8. A compound would be said to "selectively inhibit a lipoxgenase enzyme" if the concentration of inhibitor required to reduce enzyme activity by 50% was at least about 5 times less than the amount required to reduce activity of a second lipoxgenase enzyme by the same degree (under the same conditions, i.e. temperature, substrate concentration, etc.)

An "antagonist" is a compound that binds and occupies a receptor without activating it. In the presence of a sufficient concentration of antagonist, an agonist cannot activate its receptor. Therefore, antagonists may decrease the neurotoxicity mediated by NMDA (as described in Example 3) or AMPA and Kainate (as described in Example 4).

An "agonist" is a compound that activates a receptor. When the receptor is activated for a longer than normal period of time, this may cause neurotoxicity, as in the case of NMDA, AMPA and kainate receptors (see Examples 3 and 4).

The term "alkyl" refers to a cyclic, branched, or straight chain alkyl group containing only carbon and hydrogen, and unless otherwise mentioned contains one to twelve carbon atoms. This term is further exemplified by groups such as methyl, ethyl, n-propyl, isobutyl, t-butyl, pentyl, pivalyl, heptyl, adamantyl, and cyclopentyl. Alkyl groups can either be unsubstituted or substituted with one or more substituents, e.g. halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

The term "lower alkyl" refers to a cyclic, branched or straight chain monovalent alkyl radical of one to seven carbon atoms. This term is further exemplified by such radicals as methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl (or 2-methylpropyl), cyclopropylmethyl, i-amyl, n-amyl, hexyl and heptyl. Lower alkyl groups can also be unsubstituted or substituted, where a specific example of a substituted alkyl is 1,1-dimethyl heptyl.

"Hydroxyl" refers to --OH.

"Alcohol" refers to R--OH, wherein R is alkyl, especially lower alkyl (for example in methyl, ethyl or propyl alcohol). An alcohol may be either linear or branched, such as isopropyl alcohol.

"Carboxyl" refers to the radical --COOH, and substituted carboxyl refers to --COR where R is alkyl, lower alkyl or a carboxylic acid or ester.

The term "aryl" or "Ar" refers to a monovalent unsaturated aromatic carbocyclic group having a single ring (e.g. phenyl) or multiple condensed rings (e.g. naphthyl or anthryl), which can optionally be unsubstituted or substituted with, e.g., halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-

1-yl, or other functionality.

The term "alkoxy" refers to a substituted or unsubstituted alkoxy, where an alkoxy has the structure --O--R, where R is substituted or unsubstituted alkyl. In an unsubstituted alkoxy, the R is an unsubstituted alkyl. The term "substituted alkoxy" refers to a group having the structure --O--R, where R is alkyl which is substituted with a non-interfering substituent. The term "arylalkoxy" refers to a group having the structure --O--R--Ar, where R is alkyl and Ar is an aromatic substituent. Arylalkoxys are a subset of substituted alkoxys. Examples of useful substituted alkoxy groups are: benzyloxy, naphthyloxy, and chlorobenzyloxy.

The term "aryloxy" refers to a group having the structure --O--Ar, where Ar is an aromatic group. A particular aryloxy group is phenoxy.

The term "heterocycle" refers to a monovalent saturated, unsaturated, or aromatic carbocyclic group having a single ring (e.g. morpholino, pyridyl or faryl) or multiple condensed rings (e.g. indolizinyl or benzo[b]thienyl) and having at least one heteroatom, defined as N, O, P, or S, within the ring, which can optionally be unsubstituted or substituted with, e.g. halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylakyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

"Arylalkyl" refers to the groups --R--Ar and --R--HetAr, where Ar is an aryl group. HetAr is a heteroaryl group, and R is a straight-chain or branched chain aliphatic group. Example of arylaklyl groups include benzyl and furfuryl. Arylalkyl groups can optionally be unsubstituted or substituted with, e.g., halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, peperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

The term "halo" or "halide" refers to fluoro, bromo, chloro and iodo substituents.

The term "amino" refers to a chemical functionality --NR'R" where R' and R" are independently hydrogen, alkyl, or aryl. The term "quaternary amine" refers to the positively charged group --N.sup. R'R", where R'R" and R" are independently selected and are alkyl or aryl. A particular amino group is --NH2.

A "pharmaceutical agent" or "drug" refers to a chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

All chemical compounds include both the ( ) and (-) stereoisomers, as well as either the ( ) or (-) stereoisomer.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (1985) and The Condensed Chemical Dictionary (1981).

The following examples show that both nonpsychoactive cannabidiol, and psychoactive cannabinoids such as THC, can protect neurons from glutamate induced death, by a mechanism independent of cannabinoid receptors. Cannabinoids are also be shown to be potent antioxidants capable of preventing ROS toxicity in neurons.

EXAMPLE 1

Preparation of Cannabinoids and Neuronal Cultures

Cannabidiol, THC and reactants other than those specifically listed below were purchased from Sigma Chemical, Co. (St. Louis, Mo.). Cyclothiazide, glutamatergic ligands and MK-801 were obtained from Tocris Cookson (UK). Dihydrorhodamine was supplied by Molecular Probes (Eugene, Oreg.). T-butyl hydroperoxide, tetraethylammonium chloride, ferric citrate and sodium dithionite were all purchased from Aldrich (WI). All culture media were Gibco/BRL (MD) products.

Solutions of cannabinoids, cyclothiazide and other lipophiles were prepared by evaporating a 10 mM ethanolic solution (under a stream of nitrogen) in a siliconized microcentrifuge tube. Dimethyl sulfoxide (DMSO, less than 0.05% of final volume) was added to ethanol to prevent the lipophile completely drying onto the tube wall. After evaporation, 1 ml of culture media was added and the drug was dispersed using a high power sonic probe. Special attention was used to ensure the solution did not overheat or generate foam. Following dispersal, all solutions were made up to their final volume in siliconized glass tubes by mixing with an appropriate quantity of culture media.

Primary neuronal cultures were prepared according to the method of Ventra et al. (J. Neurochem. 66:1752-1761, 1996). Fetuses were extracted by Cesarian section from a 17 day pregnant Wistar rat, and the feral brains were placed into phosphate buffered saline. The cortices were then dissected out, cut into small pieces and incubated with papain for nine minutes at 37° C. After this time the tissue was dissociated by passage through a fire polished Pasteur pipette, and the resultant cell suspension separated by centrifugation over a gradient consisting of 10 mg/ml bovine serum albumin and 10 mg/ml ovomucoid (a trypsin inhibitor) in Earls buffered salt solution. The pellet was then re-suspended in high glucose, phenol red free Dulbeco's modified Eagles medium containing 10% fetal bovine serum, 2 mM glutamine, 100 IU penicillin, and 100 μg/ml streptomycin (DMEM). Cells were counted, tested for vitality using the trypan blue exclusion test and seeded onto poly-D-lysine coated 24 multiwell plates. After 96 hours, 10 μM fluoro-deoxyuridine and 10 μM uridine were added to block glial cell growth. This protocol resulted in a highly neuron-enriched culture.

EXAMPLE 2

Preparation of Astrocytes and Conditioned Media

Astrocyte conditioned DMEM was used throughout the AMPA/kainate toxicity procedure and following glutamate exposure in the NMDAr mediated toxicity protocol. Media was conditioned by 24 hour treatment over a confluent layer of type I astrocytes, prepared from two day old Wistar rat pups. Cortices were dissected, cut into small pieces, and enzymatically digested with 0.25% trypsin. Tissue was then dissociated by passage through a fire polished Pasteur pipette and the cell suspension plated into untreated 75 cm2 T-flasks. After 24 hours the media was replaced and unattached cells removed. Once astrocytes achieved confluence, cells were divided into four flasks. Media for experiments was conditioned by a 24 hour exposure to these astrocytes, after which time it was frozen at -20° C. until use. Astrocyte cultures were used to condition DMEM for no longer than two months.

EXAMPLE 3

NMDA Mediated Toxicity Studies

Glutamate neurotoxicity can be mediated by NMDA, AMPA or kainate receptors. To examine NMDAr mediated toxicity, cultured neurons (cultured

for 14-18 days) were exposed to 250 μM glutamate for 10 minutes in a magnesium free saline solution. The saline was composed of 125 mM NaCl, 25 mM glucose, 10 mM HEPES (pH 7.4), 5 mM KCl, 1.8 mM calcium chloride and 5% bovine serum albumin. Following exposure, cells were washed twice with saline, and incubated for 18 hours in conditioned DMEM. The level of lactate dehydrogenase (LDH) in the media was used as an index of cell injury.

Toxicity was completely prevented by addition of the NMDAr antagonist, MK-801 (500 nM, data not shown). However, FIG. 1A shows that cannabidiol also prevented neurotoxicity (maximum protection 88. -.9%) with an EC50 of 2-4 μM (specifically about 3.5 μM).

EXAMPLE 4

AMPA and Kainate Receptor Mediated Toxicity Studies

Unlike NMDA receptors, which are regulated by magnesium ions, AMPA/kainate receptors rapidly desensitize following ligand binding. To examine AMPA and kainate receptor mediated toxicity, neurons were cultured for 7-13 days, then exposed to 100 μM glutamate and 50 μM cyclothiazide (used to prevent AMPA receptor desensitization). Cells were incubated with glutamate in the presence of 500 nM MK-801 (an NMDAr antagonist) for 18-20 hours prior to analysis. Specific AMPA and kainate receptor ligands were also used to separately examine the effects of cannabinoids on AMPA and kainate receptor mediated events. Fluorowillardiine (1.5 μM) was the AMPA agonist and 4-methyl glutamate (10 μM) was the kainate agonist used to investigate receptor mediated toxicity. When specifically examining kainate receptor activity, cyclothiazide was replaced with 0.15 mg/ml Concanavalin-A.

Cannabidiol protection against AMPA/kainate mediated neurotoxicity is illustrated in FIG. 1B, where LDH in the media was used as an index of cell injury. The neuroprotective effect of cannabidiol was similar to that observed in the NMDA mediated toxicity model (FIG. 1A). Cannabidiol prevented neurotoxicity (maximum protection 80. -.17%) with an EC50 of 2-4 μM (specifically about 3.3 μM). Comparable results were obtained with either the AMPA receptor ligand, fluorowillardiine or the kainate receptor specific ligand, 4-methyl-glutamate (data not shown). Hence cannabidiol protects similarly against toxicity mediated by NMDA, AMPA or kainate receptors.

Unlike cannabidiol, THC is a ligand (and agonist) for the brain cannabinoid receptor. The action of THC at the cannabinoid receptor has been proposed to explain the ability of THC to protect neurons from NMDAr toxicity in vitro. However in AMPA/kainate receptor toxicity assays, THC and cannabidiol were similarly protective (FIG. 2A), indicating that cannabinoid neuroprotection is independent of cannabinoid receptor activation. This was confirmed by inclusion of cannabinoid receptor antagonist SR-141716A in the culture media (SR in FIG. 2B). See Mansbach et al., Psychopharmacology 124:315-22, 1996, for a description of SR-141716A. Neither THC nor cannabidiol neuroprotection was affected by cannabinoid receptor antagonist (FIG. 2B).

EXAMPLE 5

Cyclic Voltametery Studies or ReDox Potentials

To investigate whether cannabinoids protect neurons against glutamate damage by reacting with ROS, the antioxidant properties of cannabidiol and other cannabinoids were assessed. Cyclic voltametry, a procedure that measures the ability of a compound to accept or donate electrons under a variable voltage potential, was used to measure the oxidation potentials of several natural and synthetic cannabinoids. These studies were performed with an EG&G Princeton Applied Research potentiostat/galvanostat (Model 273/PAR 270 software, NJ). The working electrode was a glassy carbon disk with a platinum counter electrode and silver/silver chloride reference. Tetraethylammonium chloride in acetonitrile (0.1 M) was used as an electrolyte. Cyclic voltametry scans were done from 0 to 1.8 V at scan rate of 100 mV per second. The reducing ability of cannabidiol (CBD), THC, HU-211, and BHT were measured in this fashion. Anandamide, a cannabinoid receptor ligand without a cannabinoid like structure, was used as a non-responsive control. Each experiment was repeated twice with essentially the same results.

Cannabidiol, THC and the synthetic cannabinoid HU-211 all donated electrons at a similar potential as the antioxidant BHT. Anandamide (arachidonyl ethanolamide) did not undergo oxidation at these potentials (FIG. 3). Several other natural and synthetic cannabinoids, including cannabidiol, nabilone, and levanantrodol were also tested, and they too exhibited oxidation profiles similar to cannabidiol and THC (data not shown).

EXAMPLE 6

Iron Catalyzed Dihydrorhodamine Oxidation (Fenton Reaction)

The ability of cannabinoids to be readily oxidized, as illustrated in Example 5, indicated they possess antioxidant properties comparable to BHT. The antioxidant activity of BHT was examined in a Fenton reaction, in which iron is catalyzed to produce ROS. Cannabidiol (CBD) and tetrahydrocannabinol (THC) were evaluated for their ability to prevent oxidation of dihydrorhodamine to the fluorescent compound rhodamine. Oxidant was generated by ferrous catalysis (diothionite reduced ferric citrate) of t-butyl hydroperoxide in a 50:50 water:acetonitrile (v/v) solution. Dihydrorhodamine (50 μM) was incubated with 300 μM t-butyl hydroperoxide and 0.5 μM iron for 5 minutes. After this time, oxidation was assessed by spectrofluorimetry (Excit=500 nm, Emiss=570 nm). Various concentrations of cannabinoids and BHT were included to examine their ability to prevent dihydrorhodiamine oxidation.

Cannabidiol, THC and BHT all prevented dihydrorhodamine oxidation in a similar, concentration dependent manner (FIG. 4), indicating that cannabinoids have antioxidant potency comparable to BHT.

To confirm that cannabinoids act as antioxidants in the intact cell, neurons were also incubated with the oxidant t-butyl hydroperoxide and varying concentrations of cannabidiol (FIG. 5A). The t-butyl hydroperoxide oxidant was chosen for its solubility in both aqueous and organic solvents, which facilitates oxidation in both cytosolic and membrane cell compartments. Cell toxicity was assessed 18-20 hours after insult by measuring lactate dehydrogenase (LDH) release into the culture media. All experiments were conducted with triple or quadruple values at each point and all plates contained positive (glutamate alone) and baseline controls. The assay was validated by comparison with an XTT based metabolic activity assay. As shown in FIG. 5A, cannabidiol protected neurons against ROS toxicity in a dose related manner, with an EC50 of about 6 μM. The maximum protection observed was 88. -.9%.

Cannabidiol was also compared with known antioxidants in an AMPA/kainate toxicity protocol. Neurons were exposed to 100 μM glutamate and equimolar (5 μM) cannabidiol, α-tocopherol, BHT or ascorbate (FIG. 5B). Although all of the antioxidants attenuated glutamate toxicity, cannabidiol was significantly more protective than either α-tocopherol or ascorbate. The similar antioxidant abilities of cannabidiol and BHT in this chemical system (FIG. 4), and their comparable protection in neuronal cultures (FIG. 5B), implies that cannabidiol neuroprotection is due to an antioxidant effect.

EXAMPLE 7

In vivo Rat Studies

The middle cerebral artery of chloral hydrate anesthetized rats was occluded by insertion of suture thread into it. The animals were allowed to recover from the anesthetic and move freely for a period of two hours. After this time the suture was removed under mild anesthetic and the animals allowed to recover for 48 hours. Then the animals were tested for neurological deficits, sacrificed, and the infarct volume calculated. To examine the infarct volume, animals were anesthetized, ex-sanguinated, and a metabolically active dye (3-phenyl tetrazolium chloride) was pumped throughout the body. All living tissues were stained pink by the dye, while morbid regions of infarcted tissue remained white. Brains were then fixed for 24 hours in formaldehyde, sliced and the infarct volumes measured.

One hour prior to induction of ischemia 20 mg/kg of cannabidiol was administered by intra-peritoneal injection (ip) in a 90% saline:5% emulphor 620 (emulsifier):5% ethanol vehicle. A second ip 10 mg/kg dose of cannabidiol was administered 8 hours later using the same vehicle. Control animals received injections of vehicle without drug. IV doses would be expected to be 3-5 times less because of reduction of first pass metabolism.

The infarct size and neurological assessment of the test animals is shown Table 1.

TABLE 1 Cannabidiol protects rat brains from ischemia damage Volume of Infarct Behavioral Deficit (mm3) Score Animal Drug Control Drug Control 1 108.2 110.5 3 2 2 83.85 119.6 4 4 3 8.41 118.9 3 4 4 75.5 177.7 1 4 5 60.53 33.89 1 3 6 27.52 255.5 1 5 7 23.16 143 1 4 Mean 55.3 137.0 2.0 3.7 SEM 13.8 25.7 0.5 0.4 p = 0.016 significant p = 0.015 significant *Neurological scoring is performed on a subjective 1-5 scale of impairment. 0 = no impairment, 5 = severe (paralysis)

This data shows that infarct size was approximately halved in the animals treated with cannabidiol, which was also accompanied by a substantial improvement in the neurological status of the animal.

These studies with the nonpsychotropic marijuana constituent, cannabidiol, demonstrate that protection can be achieved against both glutamate neurotoxicity and free radical induced cell death. THC, the psychoactive principle of cannabis, also blocked glutamate neurotoxicity with a potency similar to cannabidiol. In both cases, neuroprotection is unaffected by the presence of a cannabinoid receptor antagonist. These results therefore surprisingly demonstrate that cannabinoids can have useful therapeutic effects that are not mediated by cannabinoid receptors, and therefore are not necessarily accompanied by psychoactive side effects. Cannabidiol also acts as an anti-epileptic and anxiolytic, which makes it particularly useful in the treatment of neurological diseases in which neuroanatomic defects can predispose to seizures (e.g. subarachnoid hemorrhage).

A particular advantage of the cannabinoid compounds of the present invention is that they are highly lipophilic, and have good penetration into the central nervous system. The volume of distribution of some of these compounds is at least 100 L in a 70 kg person (1.4 L/kg), more particularly at least 250 L, and most particularly 500 L or even 700 L in a 70 kg person (10 L/kg). The lipophilicity of particular compounds is also about as great as that of THC, cannabidiol or other compounds that have excellent penetration into the brain and other portions of the CNS.

Cannabinoids that lack psychoactivity or psychotoxicity are particularly useful embodiments of the present invention, because the absence of such side effects allows very high doses of the drug to be used without encountering unpleasant side effects (such as dysphoria) or dangerous complications (such as obtundation in a patient who may already have an altered mental status). For example, therapeutic antioxidant blood levels of cannabidiol can be 5-20 mg/kg, without significant toxicity, while blood levels of psychoactive cannabinoids at this level would produce obtundation, headache, conjunctival irritation, and other problems. Particular examples of the compounds of the present invention have low affinity to the cannabinoid receptor, for example a Ki of greater than 250 nM, for example Ki≥500-1000 nM. A compound with a Ki≥1000 nM is particularly useful, which compound has essentially no psychoactivity mediated by the cannabinoid receptor.

Cannabidiol blocks glutamate toxicity with equal potency regardless of whether the insult is mediated by NMDA, AMPA or kainate receptors. Cannabidiol and THC have been shown to be comparable to the antioxidant BHT, both in their ability to prevent dihydrorhodamine oxidation and in their cyclic voltametric profiles. Several synthetic cannabinoids also exhibited profiles similar to the BHT, although anandamide, which is not structurally related to cannabinoids, did not. These findings indicate that cannabinoids act as antioxidants in a non-biological situation, which was confirmed in living cells by showing that cannabidiol attenuates hydroperoxide induced neurotoxicity. The potency of cannabidiol as an antioxidant was examined by comparing it on an equimolar basis with three other commonly used compounds.

In the AMPA/kainate receptor dependent neurotoxicity model, cannabidiol neuroprotection was comparable to the potent antioxidant, BHT, but significantly greater than that observed with either α-tocopherol or ascorbate. This unexpected superior antioxidant activity (in the absence of BHT tumor promoting activity) shows for the first time that cannabidiol, and other cannabinoids, can be used as antioxidant drugs in the treatment (including prophylaxis) of oxidation associated diseases, and is particularly useful as a neuroprotectant. The therapeutic potential of nonpsychoactive cannabinoids is particularly promising, because of the absence of psychotoxicity, and the ability to administer higher doses than with psychotropic cannabinoids, such as THC. Previous studies have also indicated that cannabidiol is not toxic, even when chronically administered to humans or given in large acute doses (700 mg/day).

EXAMPLE 8

Effect of Cannabidiol on Lipoxygenase Enzymes

This example describes in vitro and in vivo assays to examine the effect of cannabidiol (CBD) on three lipoxygenase (LO) enzymes: 5-LO, 12-LO and 15-LO.

In vitro Enzyme Assay

The ability of CBD to inhibit lipoxygenase was examined by measuring the time dependent change in absorption at 234 nM following addition of 5 U of each lipoxygenase (rabbit 15-LO purchased from Biomol (PA), porcine 12-LO purchased from Cayman chemicals (MI)) to a solution containing 10 μM (final concentration) linoleic acid.

Enzyme studies were performed using a u.v. spectrophotometer and a 3 ml quartz cuvette containing 2.5 ml of a stirred solution of 12.5 μM sodium linoleic acid (sodium salt) in solution A (25 mM Tris (pH 8.1), 1 mM EDTA 0.1% methyl cellulose). The reaction was initiated by addition of 0.5 ml enzyme solution (10 U/ml enzyme in solution A) and recorded for 60 seconds. Lipoxygenase exhibits non-Michaelis-Menten kinetics, an initial "lag" (priming) phase followed by a linear phase which is terminated by product inhibition. These complications were reduced by assessing enzyme activity (change in absorption) over the "steepest" 20 second period in a 60 second run time. Recordings examined the absorption at 234 nm minus the value at a reference wavelength of 280 nm. Linoleic acid was used as the substrate rather than arachidonic acid, because the products are less

inhibitory to the enzyme, thereby providing a longer "linear phase".

Cell Purification and Separation

Human platelets and leukocytes were purified from buffy coat preparations (NIH Blood Bank) using a standard Ficoll based centrifugation method used in blood banks. Prior to use, cells were washed three times to eliminate contaminating cell types. Cultured rat basophillic leukemia cells (RBL-2H3) were used as a source of 5-lipoxygenase.

In vivo Determination of Lipoxygenase Activity

Cells were incubated with arachidonic acid and stimulated with the calcium ionophore A23187. Lipids were extracted and separated by reverse phase HPLC. Product formation was assessed as the area of a peak that co-eluted with an authentic standard, had a greater absorbance at 236 nm than at either 210 or 280 nm, and the formation of which was inhibited by a lipoxygenase inhibitor.

Cell pellets were triturated in DMEM culture media, aliquoted and pre-incubated for 15 minutes with 20 μM arachidonic acid and varying concentrations of cannabidiol and/or 40 μM nordihydroguaiaretic acid (a lipxygenase inhibitor). Platelets and leukocytes were also pre-incubated with 80 μM manoalide (Biomol) to prevent phospholipase A2 activation. Product formation was initiated by addition of 5 μM A23187 and incubation for 10 minutes at 37° C. At the end of the incubation, the reaction was stopped by addition of 15% 1M HCl and 10 ng/ml prostaglandin B2 (internal standard). Lipids were extracted with 1 volume of ethyl ether, which was dried under a stream of nitrogen. Samples were reconstituted in 50% acetonitrile:50% H2 O and separated by reverse phase HPLC using a gradient running from 63% acetonitrile: 37% H2 O:0.2% acetic acid to 90% acetonitrile (0.2% acetic acid) over 13 minutes.

Measurement of NMDAr Toxicity

The ability of 12-HETE (12-(s)-hydroxy-eicosatetraenoic acid, the product of the action of 12-lipoxygenase on arachidonic (eicosatetraenoic) acid) to protect cortical neurons from NMDAr toxicity was measured as described in Example 3. The 12-HETE (0.5 μg/ml) was added either during ischemia (co-incubated with the glutamate), during post-ischemia (co-incubated with the DMEM after washing the cells), or during both ischemia and post-ischemia.

Results

Using semi-purified enzyme preparations, the effect of CBD on rabbit 15-LO and porcine 12-LO was compared. As shown in FIGS. 6A and B, CBD is a potent competitive inhibitor of 15-LO with an EC50 of 598 nM. However, CBD had no effect on the 12-LO enzyme.

Using whole cell preparations, the effect of CBD on 5- and 12-LO enzymes was investigated. As shown in FIG. 7A, CBD inhibited 5-LO in cultured rat basophillic leukemia cells (RBL-2H3) with an EC50 of 1.92 μM. However, CBD had no effect on 12-LO, as monitored by the production of 12-HETE (the product of 12-LO), in either human leukocytes or platelets (FIGS. 7B and C). The leukocyte 12-LO is similar, while the platelet 12-LO is structurally and functionally different, from the porcine 12-LO used in the in vitro enzyme study.

The ability of 12-HETE to protect cortical neurons from NMDAr toxicity is shown in FIG. 8. To achieve best protection from NMDAr toxicity, 12-HETE was administered both during and post ischemia.

Therefore, CBD serves as a selective inhibitor of at least two lipoxygenase enzymes, 5-LO and 15-LO, but had no effect on 12-LO. Importantly, this is the first demonstration (FIG. 8) that the 12-LO product 12-HETE can play a significant role in protecting neurons from NMDAr mediated toxicity. Although the mechanism of this protection is unknown at the present time, 12-HETE is known to be an important neuromodulator, due to its ability to influence potassium channel activity.

EXAMPLE 9

Methods of Treatment

The present invention includes a treatment that inhibits oxidation associated diseases in a subject such as an animal, for example a rat or human. The method includes administering the antioxidant drugs of the present invention, or a combination of the antioxidant drug and one or more other pharmaceutical agents, to the subject in a pharmaceutically compatible carrier and in an effective amount to inhibit the development or progression of oxidation associated diseases. Although the treatment can be used prophylactically in any patient in a demographic group at significant risk for such diseases, subjects can also be selected using more specific criteria, such as a definitive diagnosis of the condition. The administration of any exogenous antioxidant cannabinoid would inhibit the progression of the oxidation associated disease as compared to a subject to whom the cannabinoid was not administered. The antioxidant effect, however, increases with the dose of the cannabinoid.

The vehicle in which the drug is delivered can include pharmaceutically acceptable compositions of the drugs of the present invention using methods well known to those with skill in the art. Any of the common carriers, such as sterile saline or glucose solution, can be utilized with the drugs provided by the invention. Routes of administration include but are not limited to oral, intracranial ventricular (icv), intrathecal (it), intravenous (iv), parenteral, rectal, topical ophthalmic, subconjunctival, nasal, aural, sub-lingual (under the tongue) and transdermal. The antioxidant drugs of the invention may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, lipid carriers such as cyclodextrins, proteins such as serum albumin, hydrophilic agents such as methyl cellulose, detergents, buffers, preservatives and the like. Given the low solubility of many cannabinoids, they may be suspended in sesame oil.

Given the excellent absorption of the compounds of the present invention via an inhaled route, the compounds may also be administered as inhalants, for example in pharmaceutical aerosols utilizing solutions, suspensions, emulsions, powders and semisolid preparations of the type more fully described in Remington: The Science and Practice of Pharmacy (19th Edition, 1995) in chapter 95. A particular inhalant form is a metered dose inhalant containing the active ingredient, in a suspension or a dispersing agent (such as sorbitan trioleate, oleyl alcohol, oleic acid, or lecithin, and a propellant such as 12/11 or 12/114).

Embodiments of the invention comprising pharmaceutical compositions can be prepared with conventional pharmaceutically acceptable carriers, adjuvants and counterions as would be known to those of skill in the art. The compositions are preferably in the form of a unit dose in solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, injectable and infusible solutions, for example a unit dose vial, or a metered dose inhaler. Effective oral human dosage ranges for cannabidiol are contemplated to vary from about 1-40 mg/kg, for example 5-20 mg/kg, and in particular a dose of about 20 mg/kg of body weight.

If the antioxidant drugs are to be used in the prevention of cataracts, they may be administered in the form of eye drops formulated in a pharmaceutically inert, biologically acceptable carrier, such as isotonic saline or an ointment. Conventional preservatives, such as benzalkonium chloride, can also be added to the formulation. In ophthalmic ointments, the active ingredient is admixed with a suitable base, such as white petrolatum and mineral oil, along with antimicrobial preservatives. Specific methods of compounding these dosage forms, as well as appropriate pharmaceutical carriers, are known in the art. Remington: The Science and Practice of Pharmacy, 19th Ed., Mack Publishing Co. (1995), particularly Part 7.

The compounds of the present invention are ideally administered as soon as a diagnosis is made of an ischemic event, or other oxidative insult. For example, once a myocardial infarction has been confirmed by electrocardiograph, or an elevation in enzymes characteristic of cardiac injury (e.g. CKMB), a therapeutically effective amount of the cannabinoid drug is administered. A dose can also be given following symptoms characteristic of a stroke (motor or sensory abnormalities), or radiographic confirmation of a cerebral infarct in a distribution characteristic of a neurovascular thromboembolic event. The dose can be given by frequent bolus administration, or as a continuous IV dose. In the case of cannabidiol, for example, the drug could be given in a dose of 5 mg/kg active ingredient as a continuous intravenous infusion; or hourly intramuscular injections of that dose.

EXAMPLE 10

The following table lists examples of some dibenzopyran cannabinoids that may be useful as antioxidants in the method of the present invention. ##STR13## ##STR14## Compound R19 R20 R21 R22 R23 R24 R25 R26 H 5 7-OH-Δ1 -THC CH2 OH H H H H H H C5 H11 H 6 6α-OH-Δ1 -THC CH3 α-OH H 7 6β-OH-Δ1 -THC CH3 β-OH 8 1"-OH-Δ1 -THC CH3 OH H 9 2"-OH-Δ1 -THC CH3 OH 10 3"-OH-Δ1 -THC CH3 OH 11 4"-OH-Δ1 -THC CH3 OH H 12 6α,7-diOH-Δ1 -THC CH2 OH α-OH H 13 6v,7-diOH-Δ1 -THC CH2 OH β-OH 14 1",7-diOH-Δ1 -THC CH2 OH OH H 15 2",7-diOH-Δ1 -THC CH2 OH OH H 16 3",7-diOH-Δ1 -THC CH2 OH OH H 17 4",7-diOH-Δ1 -THC CH2 OH OH 18 1",6β-diOH-Δ1 -THC CH3 β-OH OH 19 1",3"-diOH-Δ1 -THC CH3 OH OH 20 1",6α,7-triOH-Δ1 -THC CH2 OH α-OH OH H 21 Δ1 -THC-6-one CH3 =O 22 Epoxyhexahydrocannabinol CH3 (EHHC)* 23 7-oxo-Δ1 -THC CHO H 24 Δ1 -THC-7"-oic acid COOH H 25 Δ1 -THC-3"-oic acid CH3 C2 H4 COOH H 26 1"-OH-Δ1 -THC-7"-oic acid COOH OH H 27 2"-OH-Δ1 -THC-7"-oic acid COOH OH H 28 3"-OH-Δ1 -THC-7"-oic acid COOH OH H 29 4"-OH-Δ1 -THC-7"-oic acid COOH OH H 30 3",4",5"-trisnor-2"-OH-Δ1 - COOH C2 H4 OH THC-7-oic acid H 31 7-OH-Δ1 -THC-2"-oic acid CH2 OH CH2 COOH H 32 6β-OH-Δ1 -THC-2"-oic acid CH3 β-OH CH2 COOH H 33 7-OH-Δ1 -THC-3"-oic acid CH2 OH C2 H4 COOH H 34 6β-OH-Δ1 -THC-3"-oic acid CH3 β-OH C2 H4 COOH H 35 6α-OH-Δ1 -THC-4"-oic acid CH3 α-OH C3 H6 COOH H 36 2",3"-dehydro-6U-OH-Δ1 -CH3 α-OH C3 H4 COOH THC-4"-oic acid H 37 Δ1 -THC-1",7-dioic acid COOH COOH H 38 Δ1 -THC-2",7-dioic acid COOH CH2 COOH H 39 Δ1 -THC-3",7-dioic acid COOH C2 H4 COOH H 40 Δ1 -THC-4",7-dioic acid COOH C3 H6 COOH H 41 1",2"-dehydro-Δ1 -THC-3",7- COOH C2 H2 COOH dioic acid H 42 Δ1 -THC-glucuronic acid CH3 gluc.dagger. H 43 Δ1 -THC-7-oic acid COO gluc.dagger. glucuronide *Epoxy group in C-1 and C-2 positions .dagger. Glucuronide Note: R-group substituents are H if not indicated otherwise.

Chemical structures of some of the dibenzopyran cannabinoids are shown below. ##STR15## ##STR16## ##STR17##

EXAMPLE 11

Examples of Structural Analogs of Cannabidiol

The following table lists examples of some cannabinoids which are structural analogs of cannabidiol and that may be useful as antioxidants in the method of the present invention. A particularly useful example is compound CBD, cannabidiol. Compound R19 R20 R21 R22 R23 R24 R25 R26 ##STR18## ##STR19## 44 CBD CH3 H H H H H H C5 H11 45 7-OH--CBD CH2 OH 46 6α- CH3 α-OH 47 6β- CH3 β-OH 48 1"- CH3 OH 49 2"- CH3 OH 50 3"- CH3 OH 51 4"- CH3 OH 52 5"- CH3 C4 H8 CH2 OH 53 6,7-diOH--CBD CH2 OH OH 54 3",7-diOH--CBD CH2 OH OH 55 4",7-diOH--CBD CH2 OH OH 56 CBD-7-oic acid COOH 57 CBD-3"-oic acid CH3 C2 H4 COOH ##STR20## ##STR21## 58 CBN CH3 H H H H H H C5 H11 59 7-OH--CBN CH2 OH 60 1"-OH--CBN CH3 OH 61 2"-OH--CBN CH3 OH 62 3"-OH--CBN CH3 OH 63 4"-OH--CBN CH3 OH 64 5"-OH--CBN CH3 C4 H8 CH2 OH 65 2"-7-diOH--CBN CH2 OH OH 66 CBN-7-oic acid COOH 67 CBN-1"-oic acid CH3 COOH 68 CBN-3"-oic acid CH3 C2 H4 COOH Note: R-group substituents are H if not indicated otherwise.

The invention being thus described, variation in the materials and methods for practicing the invention will be apparent to one of ordinary skill in the art. Such variations are to be considered within the scope of the invention, which is set forth in the claims below.

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